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Breast cancer, like other epithelial tumors, is a highly complex and multi-factorial process. Oncogenes involved in metastasis represent a important area of exploration toward understanding the progression of this disease. This grant focuses on the role of Deleted in Colon Cancer (DCC) and E-cadherin. Ample evidence has been published implicating both in breast cancer. In the last year, we have concentrated on the regulation of expression of these molecules in breast cancer and the regulation of adhesion. Specifically, we have shown the E-cadherin promoter appears to be regulated by trans-activation, as opposed to methylation, and further localized regions of the promoter that are critical for expression. We have also shown the expression of E-cadherin protein is concordant with expression of α-catenin and β-catenin, but not with p120ctn. Its expression is frequently altered, but rarely lost in breast ductal carcinoma, and not significantly associated with outcome. We have also examined expression of Met, the HGF/SF receptor in ductal carcinoma, since it is thought to be responsible for down-regulation of adhesion. We found overexpression of Met is a highly significant, independent predictor of decreased survival in the 20-25% of cases were it is expressed.

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Introduction:

General Background:

Breast cancer, like other epithelial tumors, is a highly complex and multi-factorial process. The biological events that occur and the causes are wide ranging. Among these events, genetic alterations, both somatic and inherited, are likely to play a major role. This premise has led to a broad search of both specific genes and chromosomal regions in efforts to correlate genetic changes to tumor behavior with the goal of improving diagnostic and therapeutic tools.

Background of previous work:

Human cancers are generally thought to arise through a multi-stage evolutionary process driven by inherited and somatic mutations of genes and clonal selection of variant progeny with progressively increasing tendency toward aggressive, unregulated growth. Progression occurs largely though somatic mutation of oncogenes and tumor suppressor genes, although some mutations in these same genes are inherited, making a given individual more susceptible to developing malignancy.

The range of oncogenes, tumor suppressor genes and chromosomal locations implicated in breast cancer is broad and ever expanding. These will not be discussed here, rather we will concentrate on the DCC and E-cadherin genes and their chromosomal locations, the focus of this grant.

Both DCC and E-cadherin have been implicated as tumor suppressor genes. The phenotype of a tumor suppressor gene is tumor progression as a result of alteration or loss of expression of the normal protein encoded by these genes. Often the phenotype is expressed as a result of loss of one of the two alleles present in normal cells. This allelic loss has been termed loss of heterozygosity (LOH). LOH has been postulated to inactivate the tumor suppressor gene located in the affected chromosomal region (Knudson's Hypothesis)(Knudson,1993). A region on chromosome 18q (18q21-23) has observed to show LOH in 35-70% of breast cancers(Thompson *et al.*,1993). This region contains the DCC gene. In colorectal cancers, DCC represents a classic tumor suppressor gene, showing LOH in over 70% of cases and grossly detectable somatic gene rearrangements in 15% of the cases (Cho and Fearon,1995).

E-cadherin maps to human chromosome 16q21.1 (Berx *et al.*,1995b), a region that has also been examined for LOH. Between 30 and 50% of breast cancer cases have shown LOH on 16q(Sato *et al.*,1991). Initially this was thought to correlate with decreased protein expression as multiple publications that showed decreased expression of E-cadherin in breast cancer(Gamallo *et al.*,1993; Moll *et al.*,1993; Oka *et al.*,1993; Rimm *et al.*,1995b; Siitonen *et al.*,1996), but more recently, only lobular carcinoma seems show E-cadherin mutations (Berx *et al.*,1995a). The significance of LOH at 16q remains to be shown. It is possible other tumor suppressor genes lie within that region.

Functionally, E-cadherin is well characterized. A hypothetical cartoon of the cadherin-based transmembrane adhesion complex (figure 1) shows the components of the complex and the associated cortical cytoskeleton. E-cadherin is shown as a dimer on the basis of recent structural studies on N-cadherin that showed it functions in a dimeric form (Shapiro *et al.*,1995). The Greek letters, α and β indicate the corresponding catenins. Plakoglobin has been shown to be identical to γ-catenin and is indicated by a γ. Although we have evidence that α-catenin can bind both spectrin (Lombardo *et al.*,1994) and actin (Rimm *et al.*,1995a) and that it exists as a dimer (Koslov *et al,* 1997), the linkages shown are hypothetical. It is possible that

it not only links the cadherin complex to the cytoskeleton by also participates in actin bundling in other parts of the cell or in other cell types. The question marks indicate that it is not known if catenin/spectrin and catenin/actin interactions can occur simultaneously. There is no evidence for direct interaction between E-cadherin and spectrin, but they co-localize and are suspected to be connected by either α-catenin or some yet unknown protein. The interactions between β- and α-catenin are shown as an example of β-catenin (a member of the *Arm* family) that binds directly to both E-cadherin and α-catenin. It is not yet known exactly which other members of the *arm* family can participate in this interaction. The stoichiometry of the interactions of β-catenins and plakoglobin are unknown although there is some evidence for a single *Arm* family member per cadherin. Plakoglobin, may participate, but probably not simultaneously with β-catenin. p120ctn, the src substrate, may bind at a different site than the other members of the *arm* family. Protein tyrosine phosphatase μ is shown to indicate a direct connection to E-cadherin in some cells.

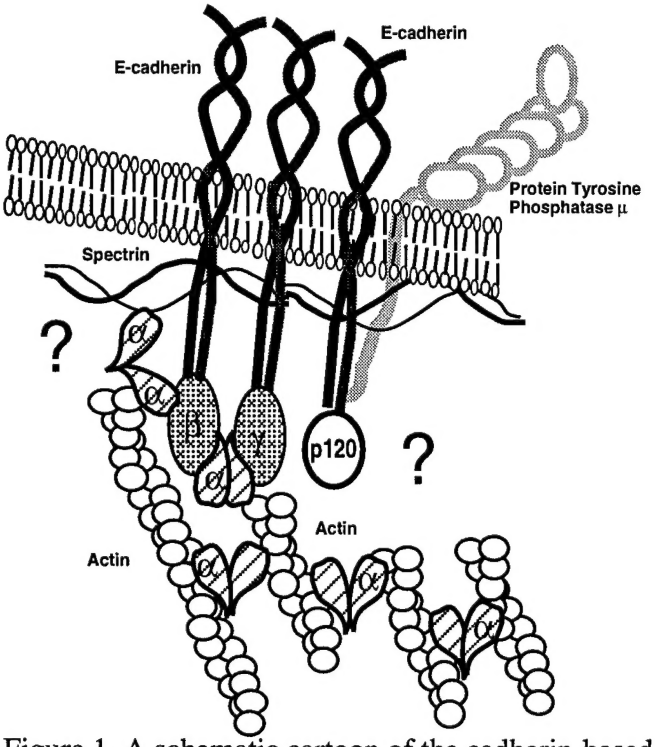

Figure 1 A schematic cartoon of the cadherin-based adhesive junction showing the proteins that interact with E-cadherin on the cytoplasmic face and their potential connections to the cytoskeleton.

Subject and Scope of Present Work:

In the original application, three technical objectives were proposed with the goal of understanding the role of the adhesion molecules DCC and E-cadherin in breast cancer. The initial task focused on use of LOH to assist in confirming that these adhesion molecules are important in the pathogenesis of breast cancer. Ultimately, the long term goals were to apply the findings of these studies to improving the diagnosis and management of breast cancer.

The following specific aims/tasks were proposed in effort to meet the above goals:

1) To determine the frequency of LOH affecting chromosomes 16q and 18q in primary breast cancers; to identify the common region affected by LOH on each of these chromosomes; and to

identify the possible associations of such LOH events with clinical and histopathological features.

2) To identify specific genetic alterations in the DCC and E-cadherin genes in breast cancers

3) To address the functional role of the DCC and E-cadherin genes as tumor suppressor genes in breast cancer

Evolution of original aims due to progress in the field:

The original aims addressed above are still largely the direction of this effort. As there has been good progress in the field on many aspects of this work, as described both above and below, there has been some modifications in our focus. Primarily, less emphasis has been placed on Task 1, as copious evidence has developed that both DCC and E-cadherin are important in the pathogenesis of breast cancer. As the ultimate goal is to improve diagnosis and management of breast cancer, we have expanded our effort on two fronts. We have increased our efforts on the proteins associated to the cytoplasmic domain of E-cadherin, with emphasis on understanding both their function and regulation. This has expanded to include growth factor receptors that have been associated with down regulation of adhesion, specifically Met, the hepatocyte growth factor/scatter factor receptor. We have also increased our efforts on understanding the regulation of expression of DCC and E-cadherin. As a result of our own work, as well as many others in the field, it is evident that mutation in these genes may represent an important, but less prominent mechanism of loss of function than alterations in the regulatory process.

Body:

SOW task 1: To determine the frequency of loss of heterozygosity (LOH) affecting chromosomes 16q and 18q in primary breast cancers; to identify the common region affected by LOH on these chromosomes; and to identify the possible associations of such LOH events with clinical and histopathological features.

Previous studies have provided evidence that LOH events affecting chromosomes 16q and 18q can be seen in a subset of breast cancers, and that the common region of LOH on 16q appears to include the E-cadherin gene and the common region of LOH on 18q includes the DCC (deleted in colorectal cancer) gene. Additional studies of LOH may serve to more precisely determine the frequency of LOH for each of these chromosomes, as well as to further localize the chromosomal region affected. In addition, the relationship of the LOH events to clinical and histopathological features of the cancers may yield important new information for assessing prognosis. Nevertheless, we have continued to emphasize studies of the leading candidate tumor suppressor genes from these chromosomes - E-cadherin on 16q and *DCC* on chromosome 18q. It should be noted that a new tumor suppressor gene from chromosome 18q21.1, termed DPC4 (for deleted in pancreatic cancer 4) has been identified. Mutations in the *DPC4* gene have been found in 40-50% of pancreatic cancers. However, recent studies suggest that *DPC4* is infrequently inactivated in other tumors, including breast cancer (Hahn *et al*,1996; Kim *et al*,1996; Schutte *et al*,1996; Thiagalingam *et al*,1996). Thus, *DCC* remains the leading candidate for the suppressor gene inactivated by 18q allelic loss in colorectal, breast, and other cancers. Finally, although our studies have provided strong supporting evidence for their inactivation in breast cancers, should we fail to obtain compelling evidence for E-cadherin and *DCC* inactivation in breast cancers, we plan to embark on studies to define the prevalence and chromosome regions affected by 16q and 18q LOH in breast cancers.

This section has been discontinued for reasons described above in the section entitled "evolution of original aims due to progress in the field".

SOW task 2a: Studies of DCC and E-cadherin gene and protein expression in breast cancer cell lines

This section was completed in Year 1. See reprint from Pierceall et al, 1995 in Oncogene, in the appendix. The DCC aspect of this task is also addressed a manuscript by Meyerhardt et al.(Meyerhardt *et al.*,1997). A reprint is included in the appendix

In light of changes in the studies outlined in the "evolution" section above, task 2a has been extended, with extensive focus on the mechanisms of E-cadherin expression. This work describes efforts to understand the E-cadherin gene expression by characterization of 5' flanking sequences and insertion of these sequences into a reporter gene (luciferase) system. Contrary to previous published work, (Graff *et al.*,1995) we find that modulation of expression is a function of trans-acting factors as opposed to changes in methylation of the promoter sequence. This work is described in detail in a paper by Ji et al (Ji *et al.*,1997) included in the appendix.

SOW task 2b: Studies of DCC, E-cadherin and α- and β-catenin in primary tumors

Expression studies were undertaken primarily by immunohistochemistry as examinations based on mRNA will only be undertaken if protein expression alterations indicate a likely result by those methods. Immunohistochemical studies have focused on E-cadherin and cadherin associated proteins including α- and β-catenin and p120ctn. This represents an extension of the

original aims as discussed in the "evolution" section above. Studies of DCC on primary tumors, although desirable, have been hampered by lack of sufficient protein levels in either tumor or normal tissue for productive immunohistochemical studies.

This work has culminated in a manuscript that has been submitted to the American Journal of Pathology. A copy of the submitted version is included in the appendix

This are of investigation was expanded over the last year to include examination of the *c-met* oncogene. It was shown by Shibamoto (Shibamoto *et al.*,1994) and others (Barth and Nelson, personal communication) that the *c-met* gene product, Met, is associated with phosphorylation of β-catenin and decreased cell-cell adhesion. Met, the receptor for HGF/SF (hepatocyte growth factor/scatter factor), is a tyrosine kinase type growth factor receptor whose stimulation results in a range of cellular changes beyond decreased cell-cell adhesion including motility and mitogenesis (Komada and Kitamura,1993) and tubulogenesis (Sachs *et al.*,1996).

Recently, examination of immuno-reactive HGF concentration in primary breast cancer tissue was shown to be an independent prognostic marker in prediction of both recurrence and survival. High concentrations of this factor were associated with shorter survival (Yamashita *et al.*,1994). However this study did not examine the stromal or epithelial source of the HGF in breast cancer tissues. Surprisingly, no follow-up study was done to assess the association between Met expression and survival. One group noted decreased immunostaining of Met in breast cancer epithelium when compared with adjacent tumor-free breast epithelium (Tsarfaty *et al.*,1992) but no association with survival or other prognostic markers was attempted. Findings of HGF mRNA expression by in-situ hybridization (Wang *et al.*,1994) and HGF and Met mRNA expression (Tuck *et al.*,1996) was seen in both benign and malignant breast (Jin *et al.*,1997).

As a part of our study examining adhesion related proteins in breast cancer, Met immunostaining was performed on tissue obtained from 91 breast resections between 5 and 15 years ago. All patients had invasive ductal breast cancer with approximately half of them presented with metastases to regional lymph nodes. All cases were examined by immunofluorescence using a Met polyclonal antibody to the cytoplasmic domain of the receptor (C12 (#sc-10) from Santa Cruz Biotech.). Most cases (78%) showed marked loss of expression of Met, while expression in adjacent normal ducts was still present. However, strong Met immunoreactivity was seen in 20 of the 91 (22%) invasive breast ductal carcinoma.

The five year survival in the in patients whose cancers lacked Met expression was 89%, in contrast to a 52% five-year survival rate in patients whose cancers expressed Met($p=0.008$). This trend is also seen in patients without lymph node metastases at presentation where Met negative cases showed 95% five year survival compared to only 62% for c-met positive cases ($p=0.006$). Multivariate analysis using the Cox Proportional Hazards Model showed Met expression is an independent predictor of survival with predictive value equivalent to that associated with lymph node status. We conclude that expression of Met in invasive ductal carcinoma of the breast is a strong, independent predictor of decreased survival and may be a useful prognostic marker to identify a subset of lymph node negative patients with more aggressive disease. This work is detailed in a manuscript that has been submitted to the journal "Cancer" and is included in the appendix.

SOW task 2c.1: Studies to identify and characterize specific mutations in E-cadherin genes

Specific efforts to find mutations in E-cadherin in human breast tumors has met with limited success elsewhere in the field. Although lobular carcinoma of the breast has been found to have some gene mutations by three groups (Kanai, et al.,1994; Berx, et al.,1995a; Candidus, et al.,1996), no mutations in E-cadherin have yet been published for any cases of ductal

carcinoma. Examination of tumors for specific mutations of E-cadherin may be done in years 3 and 4, with some modification of the original methods to reflect the successful techniques of Berx and colleagues (Berx, et al.,1995a).

We have used the more recent work of Berx and colleagues (Berx *et al.*,1996) as a guide for synthesis of PCR primers and detection mutations by SSCP. We have also shifted our focus to only lobular carcinomas, as the Berx group and others have never found mutations in ductal carcinoma, while the frequency of E-cadherin mutations in lobular carcinoma is reported as high as 50%. Our revised goal has been to examine lobular carcinomas in efforts to correlate mutations in E-cadherin with outcomes, including recurrence and survival. We have selected exons 6 through 14 for initial screening as >90% of mutations found but the Berx group are in that region. An example of assessment of PCR reactions and SSCP are shown in figure 2 below.

Our progress to date has been good with respect to amplification of each exon, but we have not yet found definitive evidence of mutation in any of the 12 cases we have examined. The results so far are summarized in table 1.

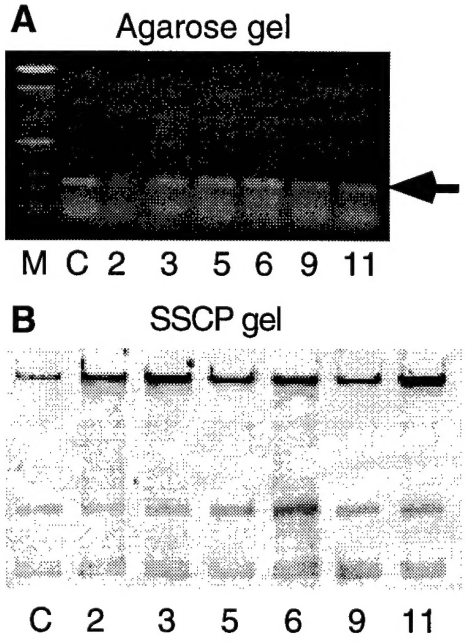

Figure 2. An agarose gel (A) shows the PCR products from the primer set for exon 11 from a control genomic DNA from human colon (C) and 6 cases of lobular carcinoma of the breast (case numbers shown beneath each lane). The lane labeled M is a 100 bp ladder marker showing that the product size (arrow) is around 250bp as expected. Part B shows the SSCP gel of the PCR products shown in part A. The normal pattern expected for this product is shown in the lane labeled C. None of these 6 cases are suggestive of mutation or polymorphism at exon 11.

Table 1. Summary of SSCP data by case number for each exon

Case#	Exon 6	7	8	9	10	11	12	13	14
Lob1	P	P	nd	nd	nd	nd	nd	nd	nd
Lob2	P	P	P	P	N	P	N	N	P
Lob3	P	P	P	P	N	P	N	N	P
Lob4	P	P	nd	nd	nd	nd	nd	nd	nd
Lob5	P	P	P	P	N	P	P	N	P
Lob6	P	P	P	P	N	P	P	N	P
Lob7	P	P	nd	nd	nd	nd	nd	nd	nd
Lob8	P	P	nd	nd	nd	nd	nd	nd	nd
Lob9	P	P	P	P	N	P	P	N	P
Lob10	P	P	nd	nd	nd	nd	nd	nd	nd
Lob11	P	P	P	P	N	P	P	N	P
Lob12	P	nd	nd	nd	nd	nd	nd	nd	nd

Key: P=product seen, no mutation; N= no PCR product; M=product seen, putative mutation in E-cadherin at that exon; nd= not yet done.

SOW task 2c.2: Alterations in *DCC* expression and gene structure in breast cancers

We have assessed DCC gene expression in a panel of breast cancer cell lines using ribonuclease (RNase) protection and reverse transcription polymerase chain reaction (RT-PCR) assays. DCC expression is not detectable by RNase protection in the cell lines studied thus far nor is it detectable by RT-PCR in the majority of cell lines studied ((Meyerhardt, et al.,1997) and unpubl. observ.). We have recently developed protocols for the detection of DCC expression by immunohistochemical approaches (Reale et al,1996) and we will use this approach to assess DCC protein expression in primary breast cancers. We have undertaken Southern blot analysis of a limited number of breast cancer cell lines using DCC cDNA probes and no alterations were found (unpubl. observ.). Because the vast majority of breast cancer cell lines lack DCC transcripts and the *DCC* exons are distributed over greater than 1.35 million basepairs at chromosome 18q21.2, we have not undertaken a sequence-based analysis of the *DCC* gene structure.

SOW task 3a: Transfection, isolation and preliminary characterization of breast cancer lines with E-cadherin, β- and α-catenin and DCC cDNAs.

Efforts toward this task are well underway but have met with limited success. We have produced cDNAs encoding all 4 proteins and successfully cloned them into a range of eukaryotic expression vectors. We have focused predominantly on the CMV driven systems and either lipofection or electroporation as mechanisms for transfection. To date we have been unable to produce and stable transformants of α-catenin or E-cadherin in any breast cancer cell lines. We have had limited success in a non-breast cancer line (Clone A) and are using this system to optimize conditions(Roe *et al.*,1996). Unfortunately, the transformation of these genes seems to confer a significant growth rate disadvantage, and although we are able to see transient expression and even stable transfection by immunofluorescence at early time points, minimal passaging of the cells to produce sufficient numbers to analyze, or even freeze, have resulted in loss of expression. The observation that α-catenin overexpression results in a dramatically decreased growth rates has been made by another lab as well and was recently published(Bullions *et al.*,1997).
To circumvent this problem, we have been construction of expression vectors and cell lines for use in the tetracycline inducible system described by Gossen and colleagues(Gossen et al,1992; Gossen *et al.*,1995).

Similar difficulties have been encountered with DCC transfection. We have undertaken transfection of two breast cancer cell lines that lack endogenous DCC expression - SK-BR-3 and BR474. As we proposed in our initial application, we have utilized three different CMV-driven mammalian vectors: pCMV/DCC-S, a vector encoding full-length DCC; pCMV/DCC-T, a vector encoding a DCC form lacking the majority of its cytoplasmic domain sequences; and pCMV/DCC-M, a control vector encoding a severely truncated form of DCC. Thus far, we have picked more than 40 G418-resistant clones following transfection of the SK-BR-3 cell line with each of the three vectors (Table 2). These clones have been expanded and Western blot analysis analyses have been carried out to identify clonal lines expressing full-length or cytoplasmic-truncated forms of DCC. None of the 15 cell lines transfected with the pCMV/DCC-S vector and analyzed by Western blot analysis have been found to express DCC. Only two of the 18 lines transfected with the pCMV/DCC-T vector stably express the truncated protein.

Table 2. Transfection of SK-BR-3 with DCC constructs

	# of Independent Colonies Reaching Indicated Point of Analysis			
Construct	24-well	T-25 Flask	Western Blot	# of DCC-Pos*
pCMV/DCC-S	43	16	15	0
pCMV/DCC-T	54	20	18	2
pCMV/DCC-M	47	17	4	0

* - DCC protein expression; note no DCC protein expression is predicted from the DCC-M construct because the predicted mutant protein would be truncated immediately downstream of the signal sequence at codon 57.

Thus far, only a relatively limited number of G418-resistant clones from transfections of the BT474 cell line have been analyzed for DCC expression, and no stably expressing clones have been obtained thus far (data shown). Because we and others have previously used the CMV-driven DCC expression vectors to generate a number of mammalian cell lines with stable expression of full-length and truncated forms of DCC (Pierceall *et al*,1994)(Klingelhutz *et al*,1995) (E.R. Fearon, unpublished observations), our findings, though preliminary, suggest that DCC expression may cause growth arrest or promote cell death in transfected breast cancer cell lines. To better address this hypothesis, we have generated constructs in which DCC cDNAs have been placed downstream of tetracycline-responsive elements (Gossen *et al*,1992). Using these constructs, we hope to generate breast cancer cell lines in which DCC expression can be tightly regulated by the levels of tetracycline in the culture media. Our preliminary studies suggest that inducible expression of DCC can be obtained following transfection with these vectors, although the vectors appear to confer somewhat leaky constitutive expression of DCC in the presence of tetracycline in several human cancer cell lines. Finally, we are in the process of generating recombinant adenoviral constructs containing the various DCC cDNAs. Because of the high efficiencies of gene transfer obtainable with adenoviral vectors, these vectors should be particularly useful for rapid assessment of the effects of DCC expression on breast cancer cell growth.

SOW task 3a: Further characterization of the *in vitro* growth properties of breast cancer lines transformed with E-cadherin, β- and α-catenin and DCC cDNAs.

Although this task is targeted for years 2-4, it is dependent on the success of task 3a, which has not yet occurred.

Conclusions:

I. Expression of E-cadherin, α-catenin and β-catenin in breast cancer cell lines is altered in a large number of cases.

Alterations are often, but not always, seen in one member of the complex where the others are expressed normally.

Alterations occur at both the protein and message levels in breast cancer cell lines.

II. Using a luciferase reporter gene, the E-cadherin promoter shows transcriptional activity in 3 breast cancer lines that express E-cadherin protein as well as 3 lines that do not.

Expression of the reporter gene in an unmethylated construct parallels that of the endogenous E-cad gene in each cell line, suggesting methylation does not down regulate the E-cad promoter Furthermore, treatment with 5-aza-2'deoxycytidine does not reactivate E-cad in lines where E-cad is not transcribed. We conclude the down regulation of transcription at the E-cad promoter is not a function of methylation but rather a trans-acting factor.

III. Unlike breast cancer cell lines, expression patterns showed high concordance for E-cadherin, α-catenin and β-catenin, but p120ctn is independently regulated in essentially all human breast cancer cases.

Altered patterns of expression are present in 80% of invasive ductal carcinomas of the breast for the three concordant antigens, but somewhat less for p120ctn. Only p120ctn shows complete loss of expression, and then only in about 10% of the cases.

High levels of concordance of expression between all three proteins and rare examples of complete loss of expression, along with the fact that 36% of cases with normal α-catenin expression are node positive (have metastasized) suggests that down regulation of the adhesion molecules (at either the transcriptional or translational level) must play a more significant role than somatic mutation in affecting loss of adhesive function.

IV. Expression of Met, the HGF/SF receptor is lost in 75-80% of breast cancers.

The 20-25% of cases that show strong expression of Met have significantly worse outcomes than those that lose expression

Expression of Met has independent predictive value for poor prognosis as assessed by the Cox Model, with a magnitude approximately equivalent to, but independent of that seen for lymph node metastasis.

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Appendix:

manuscipts:

1. Pierceall, W. E., A. S. Woodard, J. S. Morrow, D.L. Rimm and E. R. Fearon. (1995) *Frequent alterations in E-cadherin and alpha- and beta-catenin expression in human breast cancer cell lines.* Oncogene **11:**1319-26.

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Frequent alterations in E-cadherin and α- and β-catenin expression in human breast cancer cell lines

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Alterations in intercellular junction and membrane cytoskeletal proteins may underlie some of the morphological, invasive and metastatic properties of cancer. E-cadherin, a transmembrane protein that functions in epithelial cell-cell interactions at adherens junctions, is linked to the membrane cytoskeletal matrix through interactions with α- and β-catenin. We have carried out studies of *E-cadherin* and α- and β-*catenin* in 18 breast cancer cell lines to determine the prevalence and nature of alterations in these genes in breast cancer. Ten lines failed to express E-cadherin protein at detectable levels and seven failed to produce detectable levels of *E-cadherin* transcripts. In a line lacking E-cadherin expression (SK-BR-3) a homozygous deletion of a large portion of the *E-cadherin* gene was noted. Localized sequence alterations in *E-cadherin* transcripts were not identified in any lines. In contrast to the results of a previous study, no relationship was identified between *E-cadherin* expression and HER-2/NEU expression. Two of the 18 lines had no detectable α-catenin protein and six others had reduced levels. The two lines lacking α-catenin protein had reduced or undetectable levels of α-*catenin* transcripts, while no consistent changes in α-*catenin* transcript levels were seen in the lines with reduced, but detectable, levels of α-catenin protein. Similarly, although two lines lacked β-catenin protein, in most lines the levels of β-*catenin* transcripts and protein were not well correlated with one another. Our findings suggest that alterations in E-cadherin and α- and β-catenin expression are frequent in human breast cancer-derived cell lines, and that in some cases the decreased expression may result from mutations in the genes. Furthermore, the frequent alterations in the expression of these proteins argue that loss of function in the E-cadherin-catenin pathway may be critical in the development of many breast cancers.

Keywords: breast cancer; E-cadherin; α-catenin; β-catenin; cytoskeleton; cell-cell interactions

Introduction

The cadherins are a family of Ca^{2+}-dependent transmembrane proteins which appear to mediate cell-cell interactions through homotypic interactions (Takeichi, 1991; Tsukita *et al.*, 1992). E-cadherin is expressed on epithelial cells and its function depends upon the ability of its cytoplasmic sequences to link to the submembrane cytoskeletal matrix through interactions with proteins termed the catenins (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989; 1990; Kintner, 1992). The catenins include α-catenin, a protein with similarity to the actin-binding protein vinculin (Nagafuchi *et al.*, 1991; Herrenknecht *et al.*, 1991); β-catenin, a relative of the *Drosophila* armadillo protein which functions in the determination of segment polarity (McCrea *et al.*, 1991; Pfiefer and Wiechaus, 1990; Butz *et al.*, 1992) and γ-catenin, which is identical to plakoglobin and is found in both adherens junctions and desmosomal junctions (Francke *et al.*, 1989; Knudsen and Wheelock, 1992; Hinck *et al.*, 1994; Nathke *et al.*, 1994). Functional interactions between epithelial cells may be abrogated not only by defects in E-cadherin structure or expression, but also by alterations in catenin expression or structure (Hirano *et al.*, 1992).

Decreased or undetectable levels of E-cadherin expression have been noted in many immunohistochemical studies of epithelial cancers (Schipper *et al.*, 1991; Shimoyama and Hirohashi, 1991a,b; Shiozaki *et al.*, 1991; Umbas *et al.*, 1992; Brabant *et al.*, 1993; Bringuier *et al.*, 1993; Doki *et al.*, 1993; Dorudi *et al.*, 1993; Gamallo *et al.*, 1993; Mayer *et al.*, 1993; Moll *et al.*, 1993; Morton *et al.*, 1993; Oka *et al.*, 1993; Rasbridge *et al.*, 1993; Kadowaki *et al.*, 1994; Rimm *et al.*, 1995). In some tumor types, the loss of E-cadherin expression has been associated with loss of differentiated features in the tumor. In addition, the loss of E-cadherin expression in some cancers has been found to correlate with an increased likelihood of distant metastasis in the patient, suggesting a potential role for E-*cadherin* as an invasion or metastasis suppressor gene. More direct experimental support for this proposal has been obtained from in vitro studies of several rodent and human tumor cell lines in which loss of E-cadherin function is correlated with the acquisition of invasive properties (Behrens *et al.*, 1989; Frixen *et al.*, 1991; Vleminckx *et al.*, 1991; Birchmeier *et al.*, 1993).

Further evidence that loss of E-cadherin function may be critical to tumorigenesis has been provided by other studies. The chromosome 16q region containing the E-*cadherin* gene is affected by loss of heterozygosity (LOH) in breast and prostate cancers (Sato *et al.*, 1990; Bergerheim *et al.*, 1991; Carter *et al.*, 1991; Lindblom *et al.*, 1993). Somatic mutations in the E-*cadherin* gene have been identified in some gastric carcinomas, particularly diffuse type gastric cancers (Becker *et al.*, 1994; Oda *et al.*, 1994). In addition, a small subset of 130 endometrial and ovarian tumors studied were found to have somatic missense and nonsense mutations in the E-*cadherin* coding region (Risinger *et al.*, 1994). Finally, in some breast cancer lines and a

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prostate cancer cell line, evidence has been obtained that altered transcriptional regulation may account for loss of E-*cadherin* expression (Behrens *et al.*, 1991; Bussemakers *et al.*, 1994). Nevertheless, in the majority of cancers where altered E-*cadherin* expression has been observed in immunohistochemical studies, the mechanisms underlying its altered expression remain poorly understood.

Alterations in the catenins have also been seen in some human cancers, Decreased or absent α-catenin expression has been noted in some primary breast, esophageal, and prostate cancers (Shimoyama *et al.*, 1992; Morton *et al.*, 1993; Kadowski *et al.*, 1994). Genetic alterations at the α-*catenin* locus may account for decreased expression in a subset of cases. One of seven prostate cancer cell lines examined had a homozygous deletion of α-*catenin* sequences (Morton *et al.*, 1993) and a lung cancer cell line has been found to have a complete loss of α-catenin expression as a result of localized mutations in both α-*catenin* alleles (Oda *et al.*, 1993). In addition, alterations in β-catenin and plakoglobin expression and phosphorylation have also been noted in some tumor cell lines (Sommers *et al.*, 1994). Furthermore, the protein product of the adenomatous polyposis coli (*APC*) tumor suppressor gene is known to complex with α- and β-catenin, but not with E-cadherin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Hulsken *et al.*, 1994). Although the functional significance of the interactions between α- and β-catenin and the APC protein is not yet well understood, the critical role of the APC gene product in tumor suppression in epithelial cells in the gastrointestinal tract is well established (Groden *et al.*, 1991; Nishisho *et al.*, 1991). The interaction between the catenins and an established tumor suppressor gene product lends further support to the proposal that alterations in E-cadherin and catenin function may have a critical role in tumorigenesis.

In the studies described here we have sought to characterize the prevalence of alterations in E-cadherin and α- and β-catenin expression in human breast cancer-derived cell lines and to address the mechanisms underlying their altered expression. We have chosen to examine cell lines in our initial analysis to eliminate ambiguities in studies of protein and RNA expression that might have arisen as a result of the normal cells that are often admixed with neoplastic cells in primary breast cancer specimens. Complete loss or markedly decreased expression of E-cadherin and α-and β-catenin is frequently seen in breast cancer cell lines. The altered expression of E-cadherin and the catenins may result from specific mutations in the genes in some tumors and in others may reflect changes in transcriptional or post-transcriptional regulatory mechanisms. Collectively, the data support the proposal that loss of function in the cadherin-catenin pathway may play a critical role in the pathogenesis of human breast cancer.

Results

Western blot studies of E-cadherin and α- and β-catenin expression

Western blot analyses of E-cadherin, α-catenin and β-catenin expression were carried out on 18 breast carcinoma -derived cell lines (summarized in Table 1). E-cadherin protein was detectable in eight lines, and α-catenin protein was detected at varying levels in 16 lines (Figure 1). No detectable α-catenin protein was seen in two cell lines (lanes 8 and 17). Reduced levels of α-catenin were seen in six lines (lanes 3, 6 and 11–14). β-catenin protein was detected at varying levels in 16 of the cell lines. No detectable β-catenin was seen in two lines (lanes 3 and 14), and decreased levels were noted in six other cell lines (lanes 2, 8, 9, 11–13, 18). In addition, a reactive protein with aberrant migration that may represent a proteolytic breakdown product or a truncated, mutant β-catenin protein product was detected in one line (lane 10).

In an effort to determine if other proteins associated with the membrane cytoskeletal matrix might also display altered expression in the breast carcinoma-derived cell lines, we studied the expression of three

Table 1 E-cadherin, α-catenin, and β-catenin gene and protein expression in breast cell lines

		E-cadherin		*α-catenin*		*β-catenin*		*HER2/Neu*
Cell line#	*Identity*	*RNA*	*Pro*	*RNA*	*Pro*	*RNA*	*Pro*	*Pro*
1	MDA-MB-361	+ + +	+	+ +	+ + +	+ + +	+ + +	+ + +
2	BT-474	+ +	+ +	+ +	+ + +	+	+	+ + + +
3	ZR-75-30	+/–	–	+ + +	+	+ +	–	+ + + +
4	BT-20	+ +	+	+ +	+ + +	+ + +	+ + +	+
5	HBL-100	–	–	+ +	+ + +	+ +	+ +	+
6	DU4475	+	–	+	+	+ + +	+ + +	+/–
7	HS 578T	–	–	+ +	+ + +	+ +	+ + +	+
8	MDA-MB-468	+	+	+	–	+ + +	+	+
9	ZR-75-1	+ +	+ +	+ +	+ + +	+ +	+	+ +
10	BT-483	+ +	+ +	+ +	+ + +	+ +	+ +	+ +
11	MDA-MB-435S	–	–	+	+	+ +	+	+
12	MDA-MB-231	–	–	+ +	+	+ +	+	+
13	MDA-MB-453	+/–	–	+ +	+	+ +	+	+ + +
14	SK-BR-3	–	–	+ + +	+	+ +	–	+ + +
15	T-47D	+ +	+ +	+ +	+ + +	+ +	+ + +	+
16	BT-549	–	–	+ +	+ + +	+ +	+ + +	+
17	MDA-MB-157	–	–	–	–	+ +	+ +	+
18	MCF-7	+ + +	+	+ +	+ + +	+ +	+	+

Relative levels of RNA and protein expression are indicated based on RNase protection studies of gene expression and ECL-Western blot studies of protein expression, with the following scoring system: '–' no detectable expression; '+/–' very low expression; '+' low expression; '+ +' moderate expression; '+ + +' high expression; '+ + + +' very high expression

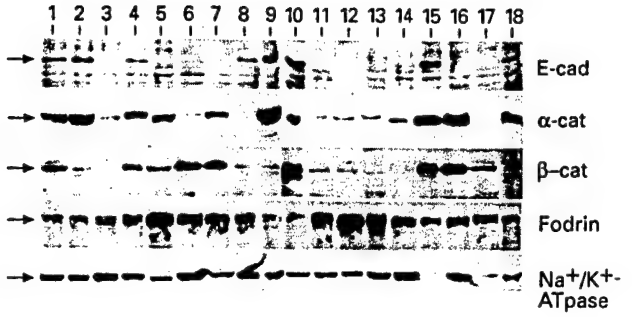

Figure 1 ECL-Western blot studies of E-cadherin and α- and β-catenin expression. Protein lysates were prepared from 18 breast cancer cell lines and 40 μg of protein from each line was loaded for SDS–PAGE on 7.5% gels. Proteins were transferred to Immobilon P membranes, and the membranes were incubated with a specific primary antiserum against E-cadherin, α-catenin, β-catenin, β-fodrin, or Na^+-K^+-ATPase and horseradish peroxidase-labeled secondary antibody reagents. Antibody complexes were detected by ECL. For each protein species studied (e.g., E-cadherin, α-catenin, etc.), although the lysates were electrophoresed on two gels and transferred to two membranes, the ECL exposure times for all 18 lanes are equivalent. The lane numbers correspond to the reference numbers in Table 1

other proteins localized to either the membrane (i.e., α-subunit of Na^+-K^+-ATPase) or the submembrane cortical cytoskeleton (i.e., βII-spectrin or β-fodrin, a spectrin-related protein that complexes with ankyrin and the band 4.1 protein which is thought to stabilize interactions between spectrin and actin). Only relatively subtle differences in β-fodrin levels were noted among the cell lines and most differences appeared to be due to decreased transfer of the gel lanes near the edges of the membrane for this large protein with a relative molecular mass greater than 200 000 (Figure 1 and data not shown). In addition, no apparent differences were noted in the expression of the band 4.1 protein in the cell lines (data not shown). However, two lines were found to have markedly reduced Na^+-K^+-ATPase α-subunit expression (lanes 15 and 17).

RNase protection studies of E-cadherin *and* α- *and* β-catenin *gene expression*

Ribonuclease (RNase) protection studies were carried out in an effort to determine if the levels of E-*cadherin* and α- and β-*catenin* transcripts were correlated with their levels of protein expression in the cell lines. Antisense riboprobes for each gene as well as a control gene (γ-*actin*) were synthesized and incubated with total RNA from each of the 18 cell lines. E-*cadherin* transcripts were detected in 11 lines (Figure 2A). Overall, the relative levels of E-*cadherin* transcripts and protein in the cell lines were well correlated (Table 1, $P<0.01$). Altered transcriptional regulation and/or mutations interfering with gene expression are likely to account for the concordant decrease in levels of E-*cadherin* transcripts and protein in the affected cell lines.

In contrast to the findings for E-cadherin, the abundance of α- and β-*catenin* transcripts in the cell lines was not well-correlated with their respective protein levels. For example, several cell lines (e.g., lanes 3, 12 and 14) had levels of α-*catenin* transcripts comparable to other lines (Figure 2B), but had very reduced levels of α-catenin protein in the Western blot analysis (Figure 1 and Table 1). The two cell lines lacking α-catenin protein by Western blot analysis had reduced or undetectable levels of α-*catenin* transcripts in the RNase protection analysis (Figure 2B, lanes 8 and 17, respectively). β-catenin protein levels were also not well correlated with transcript levels (e.g., lanes 3, 8, 12, 14 in Figures 1 and 2C; Table 1).

Southern blot and PCR-SSCP analysis of E-cadherin *gene sequences*

To determine if gross rearrangements of E-*cadherin* sequences might account for decreased or undetectable levels of E-*cadherin*, we carried out Southern blot analysis on EcoRI-digested DNA from the cell lines using a full-length E-*cadherin* cDNA probe. Changes in the migration or relative intensity of the detected fragments were seen in two cell lines (SK-BR-3, Figure 3, lane 14; and MDA-MB-468, data not shown). Based on the EcoRI fragments that failed to react with the full-length E-*cadherin* cDNA probe in the analysis of SK-BR-3, the majority of the E-*cadherin* exons in this cell line are affected by homozygous deletion (Figure 3 and data not shown). Consistent with these results, no E-cadherin protein or transcripts were detected in the SK-BR-3 line (Figures 1 and 2A, lane 14). Using an E-*cadherin* cDNA probe corresponding to exon 13–16 sequences in Southern blot analysis, we noted an EcoRI fragment with altered migration in the MDA-MB-468 cell line (data not shown). This cell line

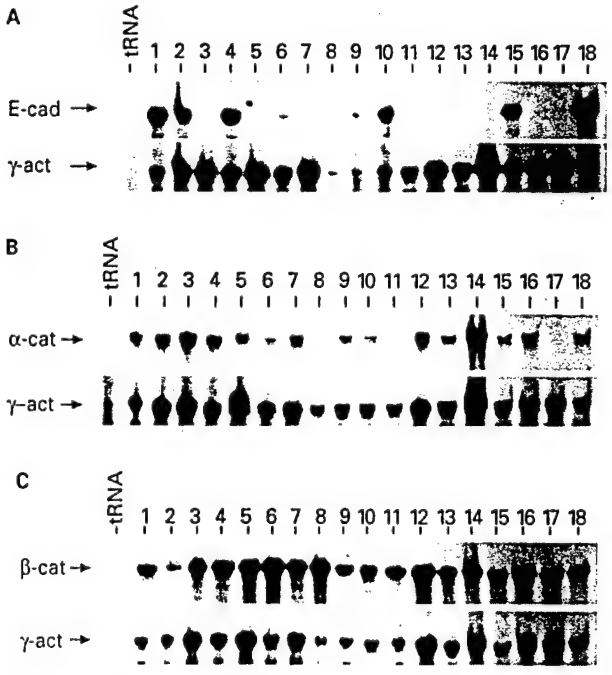

Figure 2 Ribonuclease (RNase) protection assays of E-*cadherin*, α-*catenin* and β-*catenin* gene expression. The lane numbers correspond to the reference numbers for the cell lines in Table 1 and Figure 1, with the exception of a control sample of yeast tRNA. Approximately 5 μg of total RNA from each sample was incubated with 2.5×10^4 c.p.m. of each of the acrylamide/urea gel-purified ^{32}P-labeled anti-sense riboprobes: (A) - E-*cadherin* and γ-*actin*; (B) - α-*catenin* and γ-*actin*; (C) - β-*catenin* and γ-*actin*. The γ-*actin* riboprobe was co-incubated with each of the other riboprobes to control for sample-loading and RNA integrity

expressed decreased but roughly concordant levels of E-*cadherin* transcripts and protein. Thus, the altered EcoRI-pattern seen in MDA-MB-468 following Southern analysis with E-*cadherin* cDNA probes is likely to reflect DNA polymorphism rather than mutation. No gross alterations were seen in any of the lines when a full-length α-*catenin* cDNA probe was used for Southern blot analysis of EcoRI-digested DNA from the cell lines (Figure 3 and data not shown).

Because localized mutation in E-*cadherin* sequences have previously been observed in a subset of gastric carcinomas, as well as in some ovarian and endometrial cancers, we carried out a combined polymerase chain reaction and single strand conformational polymorphism (PCR-SSCP) analysis of E-*cadherin* cDNAs obtained from cell lines in which E-*cadherin* transcripts were detected by the RNase protection assay. No sequence alterations were detected in this analysis, suggesting that localized mutations in the E-*cadherin* gene are not common in breast carcinoma cell lines expressing E-*cadherin* transcripts and protein.

Relationship between HER-2/NEU overexpression and E-cadherin expression in breast cancer cell lines

HER-2/NEU overexpression and/or amplification has been noted in a subset of breast cancers, and in several studies overexpression and/or amplification has been shown to be an independent risk factor for disease recurrence (Slamon *et al*., 1987; Press *et al*., 1993; Dougall *et al*., 1994). A recent study has suggested that overexpression of HER-2/NEU in an immortalized human mammary epithelial cell line is associated with an inhibition of E-*cadherin* transcription (D'souza and Taylor-Papadimitriou, 1994). Therefore, we sought to determine if there was a correlation between the levels of HER-2/NEU expression and E-*cadherin* expression in the breast carcinoma-derived cell lines. The relative levels of HER-2/NEU expression were characterized in the cell lines by an ECL-Western blotting approach. We found that there was no apparent correlation between E-*cadherin* expression and HER-2/NEU expression levels (Table 1 and data not shown). It has also been previously reported that E-cadherin expression was reduced but detectable in the SK-BR-3 line, a line with HER-2/NEU amplification and overexpression (D'souza and Taylor-Papadimitriou, 1994). While we confirmed that SK-BR-3 expressed high levels of HER-2/NEU, as we noted above, SK-BR-3 failed to express E-*cadherin* RNA and protein endogenously because of a homozygous deletion involving a large portion of the E-*cadherin* coding sequences.

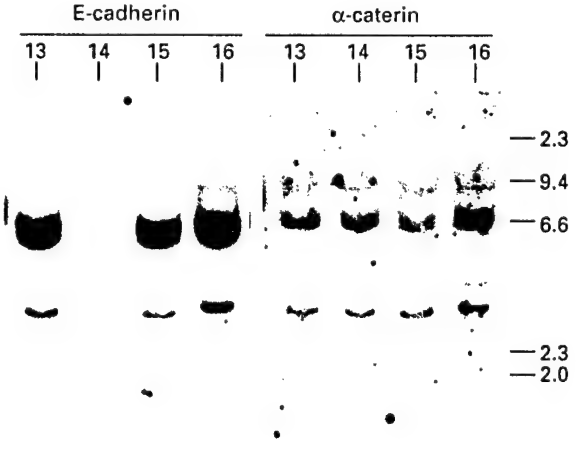

Figure 3 Southern blot analysis of EcoRI-digested breast cancer cell line DNAs with E-*cadherin* and α-*catenin* probes. The lane numbers correspond to the reference numbers for the cell lines in Table 1 and Figure 1. Approximately 10 μg of DNA from each cell line was digested with EcoRI and Southern blot analysis was carried out. The same blot was hybridized to full-length E-*cadherin* and α-*catenin* cDNAs (left and right, respectively). The SK-BR-3 cell line lost most of the EcoRI fragments detected by the E-*cadherin* cDNA probe. The migration of λ/HindIII size markers in kilobasepairs is indicated at the right

Discussion

Proper inter-cellular interactions are critical to the maintenance of normal cell morphology, differentiation, and growth control. Destabilization or loss of normal cell-cell interactions, as a result of defects in the function of the adherens junction or the submembrane cortical cytoskeleton, may have a critical role in the altered phenotype properties of cancer cells. Data supporting this proposal include the following: (i) the observations of decreased or absent E-cadherin or α-catenin reactivity in immunohistochemical studies of a number of different cancers (Schipper *et al*., 1991; Shimoyama and Hirohashi, 1991a,b; Shiozaki *et al*., 1991; Umbas *et al*., 1992; Brabant *et al*., 1993; Bringuier *et al*., 1993; Dorudi *et al*., 1993; Doki *et al*., 1993; Gamallo *et al*., 1993; Mayer *et al*., 1993; Moll *et al*., 1993; Morton *et al*., 1993; Oka *et al*., 1993; Rasbridge *et al*., 1993; Kadowaki *et al*., 1994; Rimm *et al*., 1995); (ii) the identification of mutations in the E-*cadherin* gene in a subset of gastric and gynecologic cancers (Becker *et al*., 1994; Oda *et al*., 1994; Risinger *et al*., 1994), and α-*catenin* mutations in a prostate cancer and a lung cancer cell line (Morton *et al*., 1993; Oda *et al*., 1993); (iii) the interaction of a known tumor suppressor gene product, APC, with α- and β-catenin (Rubinfeld *et al*., 1993; Su *et al*., 1993; Hulsken *et al*., 1994); (iv) the demonstration that β-catenin is phosphorylated on tyrosine residues either directly or indirectly by known oncogene products, including src, the EGF receptor and met (Hulsken *et al*., 1994); and (v) the demonstration that a transfected E-*cadherin* gene can suppress the invasive properties of some tumor cell lines with decreased or absent endogenous E-cadherin expression (Vleminckx *et al*., 1991).

In the studies described here we have addressed the prevalance of and mechanisms underlying altered E-cadherin and α- and β-catenin expression in breast cancer cell lines. Although previous immunohistochemical studies have demonstrated that E-cadherin and α-catenin immunoreactivity are each decreased or absent in about 50% of primary breast cancers (Shiozaki *et al*., 1991; Gamallo *et al*., 1993; Moll *et al*., 1993; Oka *et al*., 1993; Rasbridge *et al*., 1993; Rimm *et al*., 1995), the mechanisms underlying the altered patterns of immunoreactivity remain quite poorly characterized. In addition, while immunofluorescence studies have been carried out to address β-catenin expression in breast cancer cell lines (Sommers *et al*., 1994), no studies

addressing β-catenin immunoreactivity or expression in primary breast cancers have been presented in the literature. We have chosen to investigate the mechanisms underlying altered E-cadherin and catenin expression in breast cancer cell lines, because of some of the technical difficulties encountered in detailed studies of protein and RNA expression and gene structure and sequence in many primary breast cancers.

As summarized in Table 1, many lines had reduced or undetectable levels of expression of E-cadherin and/or the catenins. In some lines specific mutations are likely to account for the altered expression patterns. For example, a homozygous deletion of a large portion of the E-*cadherin* gene was noted in one cell line. Nevertheless, with the exception of this line, the mechanisms underlying the diminished levels of E-*cadherin* gene and protein expression noted in nearly half of the lines remain relatively poorly understood. Although the possible mechanisms include localized mutations in the E-*cadherin* gene that interfere with synthesis, processing, or stability of its transcripts, E-*cadherin* expression may be decreased as a result of specific defects in upstream regulatory pathways or transcription factors that control its expression. Indeed, consistent with this notion, previous studies suggest that E-*cadherin* promoter activity may be correlated with endogenous E-*cadherin* expression in some breast cancer cell lines (Behrens *et al*., 1991). Specific factors regulating E-*cadherin* promoter activity have not yet been well defined. Although a recent study suggested HER-2/NEU overexpression in an immortalized, non-tumorigenic breast cell line was associated with inhibition of E-*cadherin* transcription (D'souza and Taylor-Papadimitriou, 1994), in our studies, we were unable to demonstrate a correlation between HER-2/NEU overexpression and E-cadherin expression in the breast cancer cell lines.

While the relative abundance of E-*cadherin* transcripts and protein correlated well in the cell lines (Table 1), the relative levels of catenin gene and protein expression were often discordant. Specifically, although two lines with no detectable α-catenin protein expressed very reduced or undetectable levels of α-*catenin* transcripts, the majority of lines with reduced α-catenin protein had no consistent differences in α-*catenin* transcript levels. Similarly, most lines with reduced but detectable levels of β-catenin protein had no clear-cut differences in the abundance of β-*catenin* transcripts when compared to lines with abundant levels of β-catenin protein. Given that previous studies have identified α-*catenin* mutations in some cancer cell lines (Morton *et al*., 1993; Oda *et al*., 1993), a subset of the breast cancer lines with altered expression of α- or β-catenin protein may have specific mutations in the corresponding gene. An alternative, but not mutually exclusive, explanation is that an assortment of alterations in post-transcriptional and post-translational regulation of catenin expression may account for the decreased levels of catenin proteins in the cancer cell lines.

While immunohistochemical studies suggest that ductal and lobular breast cancers have some differences in the prevalence of altered E-cadherin and α-catenin expression (Ochiai *et al*., 1994), it was not clear a priori whether cell lines derived from breast cancers of differing histopathological types would display distinctly different patterns of expression. Eight of the cell lines in this study were reportedly derived from tumors with ductal histopathology (ATCC:BT-474, ZR-75-30, Hs578t, ZR-75-1, BT-483, MDA-MB-435s, T-47D, BT-549). Alterations in E-cadherin or catenin expression were seen in only a subset of these eight lines and were also seen in a similar percentage of the other lines for which the histology of the primary tumor was not noted. Thus, alterations in E-cadherin and catenin expression do not appear to be restricted to breast cancers of a particular histologic subtype.

The adhesive capacity of breast cancer-derived cell lines in Matrigel has been correlated with their expression of E-cadherin and vimentin (Sommers *et al*., 1991; Thompson *et al*., 1992). Data from those studies suggested E-cadherin expression correlated with the ability of a cell line to form spherical colonies or non-invasive clusters in Matrigel and that cell lines with high levels of vimentin expression formed invasive colonies. Nevertheless, these *in vitro* growth properties may not fully reflect appropriate function of the cadherin-catenin pathway, as one cell line (MDA-MB-468) that failed to express α-catenin protein and another cell line (SK-BR-3) lacking E-cadherin and β-catenin protein formed spherical colonies/non-invasive clusters in matrigel (Thompson *et al*., 1992).

In summary, the data presented here suggest that alterations in E-cadherin and α- and β-catenin expression are common in breast cancer cell lines. Although we cannot exclude the possibility that alterations in E-cadherin and catenin expression and gene structure may have arisen during the establishment and subsequent passage of the cell lines, the prevalence of altered expression of E-cadherin and α-catenin in the breast cancer cell lines appears to be relatively well-correlated with the results obtained from previous immunohistochemical studies of primary breast cancers. Additional studies will be necessary to further elucidate what appears likely to be a complex assortment of mutational and altered regulatory mechanisms underlying the alterations in E-cadherin and catenin expression in breast cancer cells. Moreover, while these data provide further support for the proposal that defects in the E-cadherin-catenin pathway may be a critical and necessary step in the generation of advanced breast cancer cells, definitive functional studies will ultimately be required to establish the relationship and significance of our observations to the altered phenotypic properties of breast cancer cells observed *in vivo*.

Materials and methods

Cell lines

All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in the recommended growth media. Cultures were incubated at 37°C with 5% CO_2, except for cultures maintained in Lebovitz's L-15 media which were grown at 37°C without CO_2. DNA, RNA, and protein were isolated from cultures at 75–95% confluence.

Western blot analysis

Cells were washed and then lysed in RIPA buffer [25 mM Tris-buffered saline (pH 8) with detergents (1% deoxycho-

late, 0.1% sodium dodecyl sulfate, 1% nonidet P-40) supplemented with 10 μg ml^{-1} phenylmethylsulfonyl fluoride (PMSF), 50 μg ml^{-1} antipain, 5 μg ml^{-1} aprotinin, and 2 μg ml^{-1} leupeptin (all protease inhibitors purchased from Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL), using bovine serum albumin to generate a standard curve. Forty micrograms of total protein per sample was separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Bedford, MA) by semi-dry electroblotting (Tansblot, Bio-Rad, Hercules, CA). Western blot analysis was carried out using affinity-purified polyclonal rabbit and rat or mouse monoclonal antisera as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit, goat-anti-rat or goat-anti-mouse immunoglobulin antibodies (Pierce) as secondary reagents. E-cadherin was detected with a rat monoclonal antibody DECMA-1 (Sigma). A polyclonal rabbit antiserum YR4 against α-catenin was generated by immunization with a bacterial recombinant protein containing the carboxy-terminal 447 amino acids of α-catenin fused to glutathione S-transferase (GST) (Rimm *et al.*, 1995). A rabbit polyclonal antisera against β-catenin was generated by immunization with a bacterial recombinant GST fusion protein containing full-length β-catenin sequence (D Rimm and E Koslov, unpublished observations). A polyclonal rabbit antiserum against βII-spectrin (β-fodrin) was generated by immunization with a bacterial recombinant GST fusion protein containing the carboxy-terminal third of human β-fodrin (SP Kennedy and JS Morrow, unpublished observations). The α-subunit of Na^+-K^+-adrenosine-triphosphatase (Na^+-K^+-ATPase) was detected using mouse monoclonal antibody C464.6 (a gift from Dr M Kashgarian, Dept. of Pathology, Yale University) which has been previously described (Kashgarian *et al.*, 1985). Band 4.1 protein was detected with a polyclonal rabbit antiserum raised against native band 4.1 protein purified from a red cell ghost lysate (Croall *et al.*, 1986). HER-2/Neu protein was detected by rabbit polyclonal antiserum Ab-1 (Oncogene Science, Uniondale, NY). Detection of antibody complexes was carried out with the Enhanced Chemiluminescence (ECL) Western Blot Kit (Amersham, Arlington Heights, IL) and subsequent exposure to Hyperfilm (Amersham). Western blot data shown in Figure 1 are representative of results obtained from studies performed two or more times.

Ribonuclease protection assay

Total RNA was isolated as described previously (Chomczynski and Sacchi, 1987) or using Trizol reagent (Gibco/BRL Life Technologies). Radiolabeled antisense riboprobe transcripts were prepared from plasmid constructs using T7 or T3 RNA polymerases (GibcoBRL/Life Technologies) or SP6 (New England Biolabs, Bedford, MA) and ^{32}P-CTP. Antisense riboprobes were generated from the following cDNA fragments: (i) E-*cadherin* – a 565 bp fragment containing 438 nucleotides of carboxyl-terminal coding sequences and 127 nucleotides of 3′ untranslated sequences; (ii) α-*catenin* – a 498 bp fragment corresponding to codons 449–615; (iii) β-*catenin* – a 635 bp fragment corresponding to codons 363–575; and (iv) γ-*actin* – a 275 bp fragment derived from the 3′ coding region of the cDNA. Transcripts were purified by electrophoresis, and 2.5×10^4 c.p.m. of each transcript was incubated overnight at 48°C with 5 μg of total RNA in hybridization solution [80% deionized formamide; 40 mM 1,4-piperazinediethane sulfonic acid (PIPES), pH 6.4; 400 mM NaCl; 1 mM EDTA]. The γ-*actin* transcript was co-incubated with the E-*cadherin*, α-*catenin* or β-*catenin* transcripts to control for RNA integrity and loading. Ribonuclease digestion was then carried out for 1 h at 30°C using 7.5 units of RNase T2 (Gibco BRL/Life Technologies, Grand Island, NY) in 250 μl of digestion buffer (50 mM sodium acetate, pH 4.4; 100 mM NaCl; 10 mM EDTA). RNA was precipitated with isopropanol, resuspended in RNA loading buffer (90% deionized formamide; 10 mM EDTA; 0.2% bromophenol blue; 0.2% xylene cyanol), heated for 3 min at 90°C and electrophoresed on a sequencing gel. After drying the gel, autoradiography was carried out with intensifying screens and Hyperfilm (Amersham) at –80°C. Data shown in Figure 2A–C are representative of results obtained in RNase protection assays performed two or more times.

Southern analysis

High molecular weight genomic DNA was isolated by incubation of cell pellets in 1.0% sodium dodecyl sulfate (SDS), 0.5 mg ml^{-1} proteinase K (Gibco BRL/Life Technologies) at 48°C for 24–48 h. After two extractions with phenol:chloroform:iso-amyl alcohol (50:49:1) and one extraction with chloroform:iso-amyl alcohol (49:1), DNA was recovered by ethanol precipitation. DNA concentrations were determined using a diphenylamine assay (Shatkin, 1969). For Southern analysis, 10 μg of genomic DNA was digested with EcoRI (Gibco BRL/Life Technologies), precipitated, and electrophoresed on 1.0% agarose gels. Transfer to Zeta-probe membranes (Bio-Rad) was performed using a positive pressure blotting apparatus (Posiblotter, Stratagene, San Diego, CA). Hybridization of the filters to E-*cadherin* and α-*catenin* cDNA fragments was carried out as described (Reale *et al.*, 1994). After post-hybridization washing, filters were exposed to Hyperfilm (Amersham) with intensifying screens at –80°C.

RT-PCR SSCP analysis

cDNA was prepared from DNase I-treated total RNA using random hexamers and Superscript reverse transcriptase (Gibco BRL/Life Technologies). E-*cadherin* sequences were then amplified by 35 cycles of polymerase chain reaction (PCR), with each cycle consisting of 30 s at 94°C, 45 s at 56°C, and 1 min at 72°C (last cycle 5 min). The PCR was carried out in the presence of 1.0 μCi [α-^{32}P]dCTP using standard reaction reagents and the following E-cadherin oligonucleotide pairs: pair 1 - ECAD131: 5′ - GAGAGAGGCCGCGTCCTGGGCA - 3′ and ECAD513: 5′-CCAGGTTTTTAGGAAATGGGCC-3′; pair 2 - ECAD431: 5′-CCTCAGAAGACAGAAGAGA-GAC-3′ and ECAD958: 5′-CCTGTGTTCCTGTTAATG-GTG-3′; pair 3: ECAD822: 5′-ACCTCTGTGATGGAGG TCACAG and ECAD1118: 5′-GGGATTGAAGATCGGA GGATTATC-3′; pair 4: ECAD1003: 5′-CTACGTATACC-CTGGTGGTTCA-3′ and ECAD1365: 5′-CCACATTCGT-CACTGCTACG-3′; pair 5: ECAD1473: 5′-TCCGAGGA CTTTGGCGTGGGC-3′ and ECAD1790: 5′-GAATA-TAGTTCGAGGTTCTGGTAT-3′; pair 6: ECAD1731: 5′-CTGCTGATCCTGTCTGATGTG-3′ and ECAD2113: 5′-GCAGGAATTTGCAATCCTGCTTCG-3′; pair 7: ECAD 2080: 5′ - CACAGCCTGTCGAAGCAGGATTGC - 3′ and ECAD2524: 5′-CTCAGACTAGCAGCTTCGGAACCGC-T-3′; and pair 8: ECAD2490: 5′-TATGAAGGAAGCGG-TTCCGAA-3′ and ECAD2688: 5′-ACGCTGATTTCTG CATTTCTGCAC-3′. Following amplification, 1.5 μl from each 10 μl reaction was diluted into 3.5 μl formamide sequencing stop solution, heated to 90°C for 5 min, quickly chilled on ice, and then loaded immediately onto 5% Long Ranger (AT Biochem, Malvern, PA)/10% glycerol/0.6% TBE buffer sequencing type gels and electrophoresed at 15 W for 7 h at room temperature. The gel was dried and autoradiography was carried out at –80°C with intensifying screens and Hyperfilm (Amersham).

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Identification and characterization of neogenin, a *DCC*-related gene

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***DCC* (deleted in colorectal cancer), a candidate tumor suppressor gene located in chromosome band 18q21.2, encodes a transmembrane protein of 1447 amino acids. Neogenin, a protein with nearly 50% amino acid identity to DCC, was recently identified because of its dynamic expression in the developing nervous system and gastrointestinal tract of the chicken. To explore a role for the human neogenin (*NGN*) gene in cancer, we have isolated cDNAs for two alternatively spliced forms of *NGN*, encoding proteins of 1461 and 1408 amino acids. Fluorescence *in situ* hybridization studies (FISH) localized *NGN* in chromosome band 15q22, a region infrequently affected by alterations in cancer. *NGN* transcripts of about 7.5 and 5.5 kb were detected in all adult tissues studied. In contrast to the frequent loss of *DCC* expression, no alterations in *NGN* expression were observed in more than 50 cancers studied, including glioblastoma, medulloblastoma, neuroblastoma, colorectal, breast, cervical and pancreatic cancer cell lines and xenografts. Based on their sequence conservation and similar expression during development, *DCC* and *NGN* may have related functions. However, the chromosomal location and ubiquitous expression of *NGN* in various human tumors suggest it is infrequently altered in cancer.**

Keywords: tumor suppressor gene; *DCC* (deleted in colorectal cancer) gene; brain tumor; neuroblastoma; gene expression; chromosome 18q

Introduction

Enormous progress has been made in describing genetic alterations in human cancer cells (Bishop, 1991; Knudson, 1993; Fearon, 1995). The identification of more than 50 different oncogenes has been facilitated by the prior isolation of viral oncogenes, the detection and characterization of translocation breakpoints in cancer cells, and the ability of some oncogenes to promote tumorigenic growth when transferred to nontumorigenic recipient cells. Despite much recent attention, only about 15 tumor suppressor and candidate tumor suppressor genes have been molecularly cloned (Knudson, 1993; Fearon, 1995). Results from several independent experimental approaches, however, suggest that a sizeable number of suppressor genes await discovery (Knudson, 1993; Fearon, 1995; Lasko *et al.*, 1991; Vogelstein *et al.*, 1989; Sato *et al.*, 1990; Tsuchiya *et al.*, 1992; Yamaguchi *et al.*, 1992; Seymour *et al.*, 1994; Hahn *et al.*, 1995; Cliby *et al.*, 1993; Fujino *et al.*, 1994; Ah-See *et al.*, 1994; Knowles *et al.*, 1994; Mitra *et al.*, 1994).

The existence of a tumor suppressor gene(s) on chromosome 18q was first suggested by frequent allelic losses of 18q in colorectal cancers (Vogelstein *et al.*, 1988, 1989). Subsequent studies identified the *DCC* (deleted in colorectal cancer) gene at 18q21.2 as a candidate suppressor gene (Fearon *et al.*, 1990). *DCC* is an enormous gene spanning greater than 1.35 million base pairs (bp) (Cho *et al.*, 1994), and it encodes a 1447 amino acid transmembrane protein with four immunoglobulin like and six fibronectin type III like extracellular domains, a single membrane spanning region and a 325 amino acid cytoplasmic domain (Hedrick *et al.*, 1994; Reale *et al.*, 1994). Using sensitive assays, *DCC* transcripts have been detected in most adult tissues, with highest expression seen in the developing brain and neural tube (Fearon *et al.*, 1990; Hedrick *et al.*, 1994; Reale *et al.*, 1994; Chuong *et al.*, 1994; Pierceall *et al.*, 1994; Cooper *et al.*, 1995).

DCC expression is reduced or absent in the majority of colorectal cancers, though specific somatic mutations in *DCC* have only been identified in a subset of cases (Fearon *et al.*, 1990; Cho *et al.*, 1994; Cho and Fearon, 1995). Loss of *DCC* expression has also been seen in cancers of the breast, pancreas, endometrium, prostate and brain, as well as male germ cell cancers, leukemias and neuroblastomas (Cho and Fearon, 1995; Reale *et al.*, 1996), suggesting that *DCC* inactivation may be an important factor in the development and/or progression of a variety of cancers besides those arising in the colon and rectum. A tumor suppressor function for *DCC* has, in fact, recently been demonstrated in a human squamous cancer cell line (Klingelhutz *et al.*, 1995).

A protein with roughly 50% amino acid identity to DCC, termed neogenin, was identified because of its dynamic pattern of expression in the developing nervous system and gastrointestinal tract of the chicken (Vielmetter *et al.*, 1994). Specifically, neogenin was induced in neural cells immediately prior to cell cycle withdrawal and terminal differentiation. To further our understanding of NGN and explore the possibility that alterations in neogenin might be present in cancers, we have cloned the human neogenin (*NGN*) gene. In contrast to *DCC*, based on its pattern of expression in cancer cells and its chromosomal location, *NGN* appears to be infrequently altered in cancer.

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Results

Identification of NGN, *a* DCC-*related gene*

Given the frequent loss of *DCC* expression in many types of cancer, we sought to determine if *DCC*-related genes were also inactivated in cancer. A human expression sequence tag (T07322) with over 85% nucleotide identity to chicken neogenin, a *DCC*-related gene, was identified in the GenBank database. Using this sequence as a hybridization probe, we isolated cDNA clones from a fetal brain library spanning 5297 bp and containing the entire human neogenin (*NGN*) open reading frame (Figure 1). Two alternatively spliced forms of neogenin were previously identified in the chicken, the isoforms differ by the presence or absence of a 159 bp sequence in the neogenin cytoplasmic domain. We identified both alternatively spliced forms. NGN protein products of 1461 and 1408 amino acids were predicted from the sequences (Figure 1).

The extracellular domain of human NGN displayed features common to members of the neural cell adhesion molecule (N-CAM) family, with four immunoglobulin like and six fibronectin type III (FN III) like domains. Eight potential asparagine (N)-linked glycosylation sites (N-X-S/T) were identified in the approximately 1100 amino acid extracellular region (Figure 1). A single hydrophobic membrane-spanning sequence was found. In the long isoform of *NGN*, a cytoplasmic domain of 338 amino acids with 14 potential phosphorylation sites was observed (Figure 1). Three of the sites are lost in the alternatively spliced short *NGN* isoform. Overall, the predicted amino acid sequence of human NGN was 86% identical to the chicken neogenin sequence, with the greatest similarity seen in the FN III and cytoplasmic domains (Table 1). Comparison of human NGN and DCC revealed that the proteins had identical domain structure and roughly 50% identity at the amino acid level (Table 1). The cytoplasmic sequences of NGN and DCC were less well-conserved, with only about 37% identity at the amino acid level. However, their cytoplasmic domains do not share extensive similarity with any other proteins in the database.

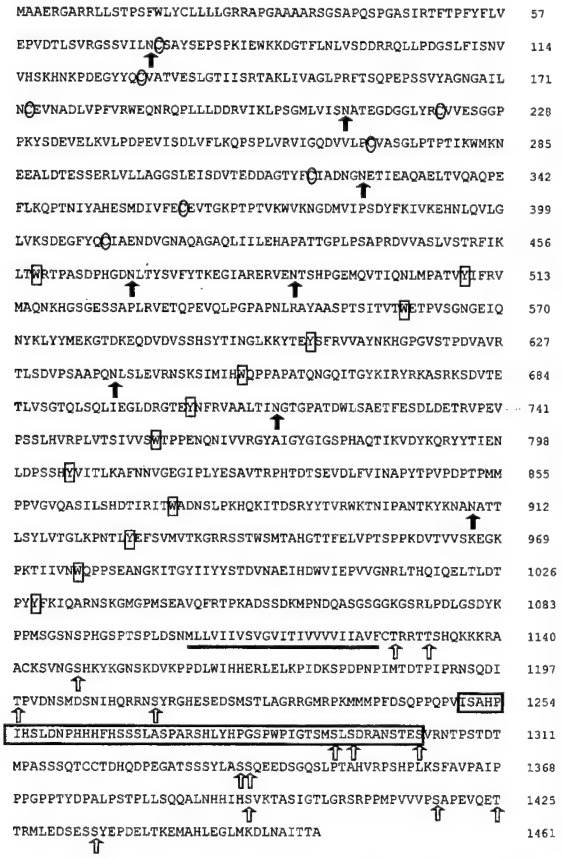

Figure 1 The predicted 1461 amino acid sequence (in single letter code) of NGN. The eight cysteines (C) in the four immunoglobulin like domains are marked by circles, and the conserved tryptophan (W) and tyrosine (Y) residues in the six FN type III domains are boxed. The eight potential N-linked glycosylation sites in the extracellular domain are indicated by solid arrows; the presumed membrane-spanning region is underlined; and the 14 potential phosphorylation sites in the cytoplasmic domain are indicated by open arrows. The sequences in the NGN cytoplasmic domain, absent in the alternatively spliced form, as boxed

NGN proteins

To demonstrate that the *NGN* cDNAs encoded proteins, Western blot analysis was performed with lysates of Cos-1 cells that had been transiently transfected with expression constructs encoding the two alternatively spliced forms of *NGN*. To facilitate their detection, vesicular stomatitis virus glycoprotein (VSV-G) epitope tags were fused to the carboxy-termini of each protein. Both cDNAs encoded proteins migrating at about 190 kDa, with the shorter NGN isoform migrating slightly more rapidly (Figure 2). Given the eight potential N-linked glycosylation sites in the NGN extracellular domain and the fact that the 1447 amino acid DCC protein migrates at roughly 175–190 kDa (Hedrick *et al*., 1994; Reale *et al*., 1994), the apparent molecular masses were in good agreement with those predicted from the *NGN* sequences.

Chromosomal localization of NGN

Data from allelic loss studies of various cancers suggest that a number of chromosomal regions contain novel

Table 1 Sequence comparisons of the human NGN, chicken neogenin and human DCC proteins

	% Amino acid identity with human NGN[a]
Chicken neogenin[b]	
Total protein	85.8%
Ig-like domains (4)	75.5%
FN type III domains (6)	90.0%
Transmembrane domain	91.3%
Cytoplasmic domain	88.5%
Human DCC	
Total protein	49.5%
Ig-like domains (4)	46.6%
FN type III domains (6)	57.3%
Transmembrane domain	60.9%
Cytoplasmic domain	37.3%

[a]Comparison with the long isoform of human NGN. [b]Comparison to amino-terminal region of chicken neogenin based on the published sequence (Vielmetter *et al*., 1994) which appears to begin at amino acid 35 when aligned with the human *NGN* sequence

tumor suppressor genes awaiting identification (reviewed in Fearon, 1995 and Lasko *et al*., 1991). Using fluorescence *in situ* hybridization, we observed specific hybridization of a human NGN P1 clone to all four 15q chromatids in 20 of 20 metaphase spreads analysed (example shown in Figure 3). In these 20 metaphases, there were no signals on any other chromosome. Localization of *NGN* to band 15q22 was based on the position of the fluorescence signals relative to chromosome landmarks. In previous studies, allelic losses of 15q have been infrequently observed in cancer (Lasko *et al*., 1991; Vogelstein *et al*., 1989; Sato *et al*., 1990; Tsuchiya *et al*., 1992; Yamaguchi *et al*., 1992; Seymour *et al*., 1994; Hahn *et al*., 1995; Cliby *et al*., 1993; Fujino *et al*., 1994; Ah-See *et al*., 1994; Knowles *et al*., 1994; Mitra *et al*., 1994). Though one recent study suggested that 15q allelic losses were common in metastatic cancers of the breast, colon and lung, the losses were restricted to proximal 15q and did not include the 15q22 region (Wick *et al*., 1996). Hence, based on its location and the allelotype studies carried out thus far, *NGN* is not likely to be frequently affected by allelic losses in cancer.

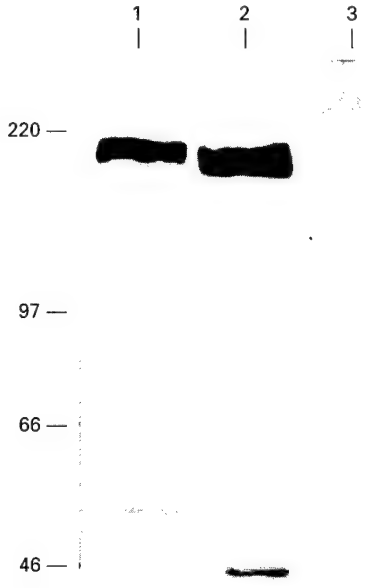

Figure 2 Western blot detection of NGN proteins. Lysates were prepared from Cos-1 cells transfected with pcDNA3 mammalian expression vectors containing *NGN* cDNAs for the long (lane 1) or short (lane 2) isoforms, each tagged with a VSV-G epitope, or a pcDNA3 vector lacking a cDNA insert (lane 3). NGN proteins appeared to migrate at roughly 190 kDa, with the shorter NGN isoform migrating slightly faster. The relative mobility of prestained marker proteins is indicated at the left (in kDa)

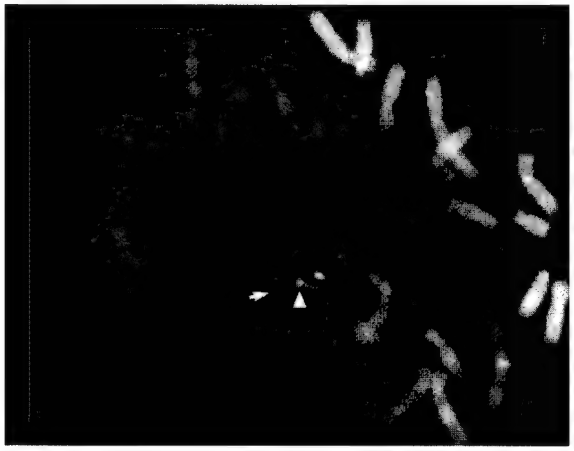

Figure 3 *NGN* maps to chromosome 15q22. (**a**) The hybridization of a *NGN* P1 clone (arrow) and a chromosome 15 centromere probe (arrowhead) to human metaphase chromosomes is shown

NGN *and* DCC *expression in normal and neoplastic tissues*

Using Northern blot analysis, *NGN* transcripts of about 7.5 and 5.5 kb were detected in all normal adult tissues studied (Figure 4a and data not shown). In

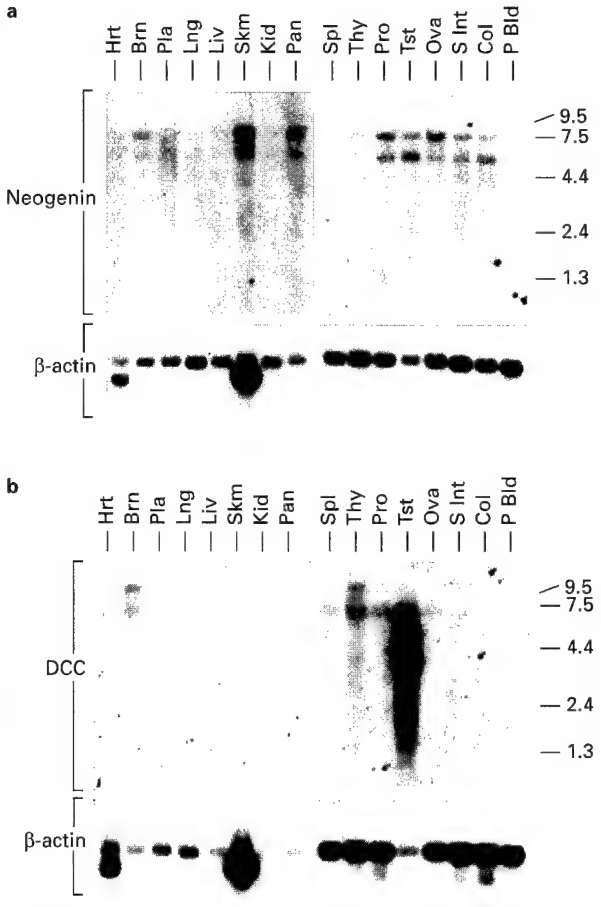

Figure 4 Northen blot analysis of *NGN* and *DCC* expression. Northern blots containing approximately 2 μg of Poly(A)$^+$ RNA in each lane were hybridized to *NGN* (**a**) or *DCC* (**b**) cDNA probes. Following hybridization to *NGN* or *DCC*, the blots were stripped and rehybridized with a β-actin cDNA probe. The lanes contain RNA from heart (Hrt), brain (Brn), placenta (Pla), lung (Lng), liver (Liv), skeletal muscle (Skm), kidney (Kid), pancreas (Pan), spleen (Spl), thymus (Thy), prostate (Pro), testis (Tst), ovary (Ova), small intestine (S Int), colon (Col) and peripheral blood cells (P Bld). The mobility (in kb) of molecular weight markers is indicated at the right

previous studies, *DCC* transcripts have been detected in most normal adult tissues (Fearon *et al*., 1990; Reale *et al*., 1994). However, their very reduced abundance has often necessitated very sensitive detection methods, such as reverse transcription polymerase chain reaction (RT–PCR). Although previous studies have suggested that *DCC* transcripts were only detectable by Northern blot analysis in adult brain (Fearon *et al*., 1990; Cooper *et al*., 1995), we were able to detect *DCC* transcripts of about 10 kb and/or 7 kb in many adult tissues studied (Figure 4b and data not shown). Of note, in testis, we also detected abundant levels of *DCC* transcripts of altered size (5.5 and 4.0 kb). These altered DCC transcripts have not been characterized in detail.

We carried out ribonuclease (RNase) protection studies to determine the relative abundance of *NGN* and *DCC* transcripts in cancers. In glioblastomas, *NGN* expression was detected in all specimens studied (Figure 5, Table 2), while *DCC* expression was not detected in upwards of 40% of the specimens (Table 2), confirming previous results (Ekstrand *et al*., 1995). *NGN* expression was also detected in all seven medulloblastoma xenografts studied, but *DCC* expression was absent in two of the seven tumors (Table 1). In additional RNase protection and RT–PCR studies, *NGN* expression was detected in all other cancer lines studied, including neuroblastoma, breast, colorectal, pancreatic, and cervical cancers (Figures 5 and 6, Table 2). In contrast, *DCC* expression was not detectable in the majority of these lines (Figure 6 and Table 2).

Discussion

Neogenin, a protein with roughly 50% amino acid identity to DCC was first identified because of its dynamic regulation in the developing nervous system and gastrointestinal tract of the chicken (Vielmetter *et al*., 1994). To further explore the role of the DCC/Neogenin family of proteins in cancer, we have cloned the human Neogenin (*NGN*) gene and localized it to chromosome 15q22. *NGN* encodes a 1461 amino acid protein, with similar structure to DCC and 50.2% amino acid identity. *NGN* transcripts were found to be expressed in all normal adult tissues studied. In

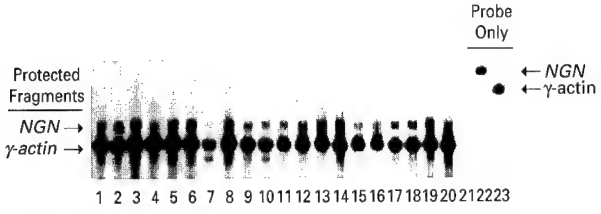

Figure 5 Ribonuclease (RNase) protection assay of *NGN* expression in cancer. Samples were glioblastoma xenografts (lanes 1–10) and colorectal (lanes 11–14), breast (lanes 15 and 16), and neuroblastoma (lanes 17–20) cancer cell lines. Ten μg of RNA from a negative control rat cell line, Rat1, was loaded in lane 21. Approximately 500 c.p.m. of the undigested 425 bp *NGN* and 375 bp γ-actin riboprobes were loaded in lanes 22 and 23, respectively. The relative mobilities of the protected 312 bp *NGN* and 275 bp γ-actin fragments are indicated. The specific xenografts and cells lines were: lane 1 - 397; lane 2 - 398; lane 3 - 408; lane 4 - 409; lane 5 - 425; lane 6 - 443; lane 7 - 493; lane 8 - 542; lane 9 - 561; lane 10 - 566; lane 11 - DLD1; lane 12 - LoVo; lane 13 - WIDR; lane 14 - Hct 116; lane 15 - SKBR3; lane 16 - MDA-MB-361; lane 17 - SJNB-14; lane 18 - SJNB-17; lane 19 - SJNB-20; lane 20 - IMR32

Table 2 *NGN* and *DCG* expression in tumor xenografts and cell lines[a]

Xenograft/Cell line	*NGN*	*DCC*
Glioblastomas		
54	++	+/–
245	++	++
259	+++	–
270	++	+/–
317	++	+
320	+++	–
368	++	++
397	++	–
398	+++	++
408	+++	++
409	++	+
443	+++	+
493	++	–
542	+++	+
561	++	+
566	++	–
640	++	+/–
Medulloblastomas		
341	++	+
384	+++	–
425	+++	+
487	++	–
511	+++	+
556	+++	+
690	+++	++
Neuroblastomas		
SJNB-7	++	+/–
SJNB-8	+++	+/–
SJNB-10	++	–
SJNB-11	++	–
SJNB-17	++	+
IMR32	++	++
Colorectal		
DLD1	+++	+/–
LoVo	+++	+
WIDR	+++	–
Hct116	+++	–
RKO	+++	+
Breast		
SKBR3	+++	–
MDA-MB-361	+++	–
MDA-MB-231	+++	–
Pancreatic		
AsPC1	+++	+
CAPAN2	+++	–
Panc1	+++	+
Su86.86	+++	–
P×4	+++	–
P×26	+++	–
P×117	++	–
Cervical		
HeLa	+++	+
HT3	+++	–
SiHA	+	–
Caski	+++	–
C4II	++	+/–
C33A	+++	+

[a]Expression studied in the majority of samples by RNase protection as well as RT–PCR with two sets of *NGN* primers and two sets of *DCC* primers (see Figures 5 and 6). The relative levels of *NGN* and *DCC* expression in the tumor specimens were scored using the following system: (–) no expression detected by either RNase protection and/or RT–PCR studies; (+/–) no detectable expression by RNase protection, but faint RT–PCR signals detected; (+) low level expression detected by RNase protection and/or RT–PCR; (++) moderate expression detected by RNase protection and/or RT–PCR; (+++) high level expression detected by RNase protection and/or RT–PCR

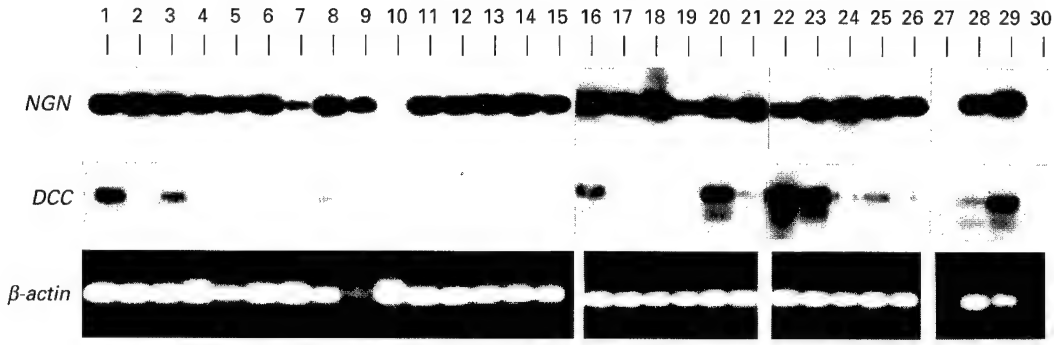

Figure 6 RT–PCR assay of *NGN* and *DCC* expression in cancer. Shown are Southern blots of the RT–PCR products generated with *NGN* cytoplasmic domain and *DCC* extracellular domain primers, and ethidium bromide-stained β-actin RT–PCR products. Pancreatic cancer cell lines and xenografts in lanes 5–7, respectively; cervical cancers in 8–13; breast cancers in 14 and 15; colorectal cancers in 16–18; neuroblastomas in 19 and 20; glioblastomas in 21–26; and control samples in lanes 27–30. The specific lines were: lane 1 - AsPC1; lane 2 - CAPAN2; lane 3 - Panc 1; lane 4 - Su86.86; lane 5 - Px4; lane 6 - Px26, lane 7 - Px117; lane 8 - HeLa; lane 9 - HT3; lane 10 - SiHA; lane 11 - Caski; lane 12 - C4II; lane 13 - C33A; lane 14 - MDA-MB-231; lane 15 - MDA-MB-361; lane 16 - LoVo; lane 17 - Hct116; lane18 - DLD1; lane 19 - SJNB7; lane 20 - IMR32; lane 21 - GBM 54; lane 22 - GBM 398; lane 23 - GBM 408; lane 24 - GBM 640; lane 25 - GBM 317; lane 26 - GBM 270; lane 27 - no RNA (negative control for RT and PCR); lane 28 - liver cDNA library; lane 29 - fetal brain cDNA library; lane 30 - no cDNA (control for PCR). The exposure times of all *NGN* lanes was 2 h and the exposure time of all *DCC* lanes was 6 hours

addition, NGN transcripts were also detected in virtually all cancer tissues and cell lines studied, including many tumors in which *DCC* expression was not detected.

Loss of *DCC* expression in cancers does not establish that *DCC* is a tumor suppressor gene. Nonetheless, the data are consistent with the possibility. Unfortunately, because of its very large size (i.e. >1.35 million bp), definitive examination of the *DCC* locus for alterations affecting its expression in cancer cells, such as somatic mutations or increased methylation of its regulatory sequences, has not been possible. In contrast to *DCC*, the chromosomal location and ubiquitous expression of *NGN* in cancer suggest that it is unlikely to be a suppressor gene. The findings presented here also imply that, despite their extensive sequence similarity, the biologic functions of *DCC* and *NGN* in cell growth regulation and cancer may be distinct.

Recent studies of genes regulating cell migration and axon guidance in the developing nervous system have provided interesting new clues into *DCC* and *NGN* function. The *C. elegans unc-40* gene encodes a transmembrane protein with identical domain structure to DCC and NGN and about 25% amino acid identity with each (Chan *et al*., 1995). Though it may also have other functions, *unc-40* is necessary for the appropriate circumferential migration of a subset of cells and axons in the developing nematode (Hedgecock *et al*., 1990). *unc-40* is believed to function in the same pathway as another gene termed *unc-6* (Hedgecock *et al*., 1990; Culotti, 1994). *unc-6* encodes a secreted protein bearing significant similarity to the amino-terminal region of the B2 chain of laminin, an extracellular matrix protein (Ishii *et al*., 1992) and two vertebrate homologues of *unc-6*, termed netrin-1 and netrin-2 have recently been identified (Serafini *et al*., 1994; Kennedy *et al*., 1994). The netrin proteins were initially identified and purified because of their ability to promote the outgrowth of commissural axons, but they also appear to function as chemoattractants for commissural axons (Kennedy *et al*., 1994; Kennedy and Tessier-Lavigne, 1995). Given the predicted similarity of UNC-40 to DCC and NGN, the findings suggests that *DCC* and *NGN* may play important roles in mediating directional cell migration in the developing nervous system.

What role, if any, would *DCC*, *NGN* and the netrins be expected to play in other tissues? Moreover, how would loss of *DCC* function contribute to the altered phenotype of cancer cells, particularly if *NGN* function is retained in the cells? As shown above, *DCC* and *NGN* are both expressed at low levels in virtually all adult tissues. We have cloned the human netrin-1 gene and have found it expressed in all adult tissues surveyed (Meyerhardt *et al*., unpublished observations). In addition, netrin-1 transcripts can be detected in many human cancer cell lines, including those derived from colorectal tumors (Meyerhardt *et al*., unpublished observations). The effects of netrins on epithelial cells are poorly understood. However, it is tempting to propose that netrins may provide growth inhibitory or differentiation cues to epithelial cells. Given the substantial differences between the DCC and NGN cytoplasmic sequences, cancer cells that have lost *DCC* function may fail to respond appropriately to netrin signals, despite retaining *NGN* expression. Alternatively, *DCC* alterations may contribute to defects in the migratory properties of cancer cells or their failure to respect tissue boundaries. Indeed, such an effect might account for the apparently more aggressive and metastatic growth properties of some cancer cells lacking *DCC* expression (Cho and Fearon, 1995; Reale *et al*., 1996).

Materials and methods

Cloning of NGN

A 312 basepair (bp) polymerase chain reaction (PCR) product corresponding to a human expression sequence tag (T07322) with 85% identity to chicken neogenin was amplified from an oligo-dT primed human fetal brain

library (Stratagene Cloning Systems, La Jolla, CA) using two oligos (5′-TTACGCCATTGGTTATG-3′ and 5′-CACCATCAGGATTACGTG-3′) derived from the ends of the sequence tag. The PCR product was labeled with [^{32}P]dCTP by random priming and used to screen the fetal brain library. Approximately 2×10^6 plaques were lifted onto Hybond N$^+$ nylon filters (Amersham, Arlington Heights, IL). The filters were hybridized and washed as described (Vogelstein *et al.*, 1987). A total of 19 independent clones were isolated by multiple rounds of hybridization selection. Phagemids were rescued by *in vivo* excision with the ExAssist/SOLR system provided with the library.

DNA sequencing

Both strands of overlapping, double-stranded phagemid clones, containing the entire open reading frame of *NGN* (GenBank #U61262), were sequenced in their entirety using a combination of external and internal primers and exonuclease III/mung bean nuclease deletions (Stratagene). Plasmid DNA was prepared using Qiagen Spin Plasmid Kit (Qiagen, Inc., Chatsworth, CA) and sequenced by the dideoxy chain termination method using Sequenase 2.0 (U.S. Biomedical Corp., Cleveland, OH) and a modified protocol (Kraft *et al.*, 1988). Sequencing reactions were electrophoresed on 6% polyacrylamide (19 : 1 acrylamide : bis-acrylamide)/8.0 M urea/1 × TBE gels. After drying, the gels were exposed to X-OMAT film (Eastman Kodak, Rochester, NY).

NGN *expression constructs*

Full-length cDNAs encoding each of the two alternatively spliced forms of *NGN* were constructed from the overlapping fetal brain cDNAs. PCR was used to fuse a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag (YTDIEMNRLGK) to the carboxyterminus of each of the two full-length *NGN* cDNAs. The modified cDNAs were sequenced to verify that no errors had been introduced. The tagged cDNAs were subcloned into the pcDNA3 mammalian expression vector (Invitrogen Corp., San Diego, CA).

Western blot analysis

Transfections of Cos-1 cells (American Type Culture Collection [ATCC], Rockville, MD) were performed with Lipofectamine (Gibco BRL Life Technologies, Gaithersburg, MD) per the manufacturer's instructions. Protein extracts were prepared from the cells 48 h after transfection as previously described (Pierceall *et al.*, 1994; Ekstrand *et al.*, 1995). Following electrophoresis on an 8% SDS-polyacrylamide gel and transfer to Immobilon-P membranes (Millipore, Bedford, MA) with a semidry electroblotter (Bio-Rad, Hercules, CA), tagged proteins were detected with a polyclonal rabbit anti-VSV-G antiserum (MBL International, Watertown, MA) and a donkey anti-rabbit IgG coupled to horseradish peroxidase (Pierce Biochemicals, Rockford, IL). Antibody complexes were detected by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL) and subsequent exposure to Kodak X-OMAT film.

P1 clone isolation and fluorescence in situ *hybridization (FISH)*

The p1 library (DMPC-HFF#10) was screened by Genome Systems, Inc. (St Louis, MO) with two *NGN* primers derived from sequences at the end of the coding region and the downstream 3′ untranslated region (nucleotides 4465–4645; sense oligo 5′-GAGATGGCCCACCTGGAAGGAC-3′ and antisense oligo 5′-GTCTGCTGGCTGATTCTG-TGTT-3′). Three P1 clones were isolated (DMFC-HFF#1-113-H12, -531-A11 and -1421-D6). Purified DNA from clone #113-H12 was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation. Phytohemagglutinin-stimulated human peripheral blood lymphocytes from a normal donor were used as the source of metaphase chromosomes. Labeled DNA was hybridized overnight at 37°C to fixed metaphase chromosomes in a solution containing sheared human DNA, 50% formamide, 10% dextran sulfate, and 2 × SSC. Specific hybridization signals were detected by incubating the slides with fluorescein-conjugated sheep anti-digoxigenin antibodies (Boehringer Mannheim). The chromosomes were then counterstained with 4,6-diamidino-2-phenylindole (DAPI) and analysed. The assignment of *NGN* to chromosome 15 was confirmed by co-hybridization of the *NGN* P1 clones and a biotinylated chromosome 15 centromere-specific probe (D15Z) (Oncor, Inc., Gaithersburg, MD). In this case, probe signals were detected by incubating the slides with fluorescein-conjugated sheep anti-digoxigenin antibodies as well as a Texas red avidin conjugate (Vector Laboratories, Burlington, CA). The *NGN* gene was further localized on chromosome 15 by comparing the position of fluorescein signals to chromosome landmarks, such as the centromere, telomere, and heterochrome and euchromatin boundaries (Franke, 1994).

RNA isolation

Brain tumor xenografts were established from primary human glioblastomas and medulloblastomas and propagated in nude mice as previously described (Schold *et al.*, 1983). Pancreatic xenografts were established from pancreatic adenocarcinomas (Hahn *et al.*, 1995). Human neuroblastoma cell lines SJNB-7, -8, -10, -11 and -17 were derived from advanced stage primary tumors at St. Jude Children's Research Hospital. All other cell lines were purchased from ATCC. Total RNA was isolated from minced brain tumor xenograft tissues or pelleted tumor cells, using Trizol reagent (Gibco BRL Life Technologies) or the RNAgents RNA isolation system (Promega, Madison, WI).

Northern analysis

Northern blots of normal human adult tissues (approximately 2 μg of poly(A)$^+$ RNA loaded per lane) were purchased from ClonTech (ClonTech Laboratories, Inc., Palo Alto, CA). Hybridization were performed according to the manufacturer's instructions using a 486 bp ^{32}P-labeled *NGN* cDNA probe (corresponding to amino acids 330–491) or a 4.35 kb ^{32}P-labeled *DCC* cDNA probe (Hedrick *et al.*, 1994). Following hybridization, blots were washed with 2 × SSC/0.5% SDS for 45 min at room temperature, with a subsequent increased stringency wash of 0.1 × SSC/0.1% SDS for 30 min at 50°C. Blots were stripped per the manufacturer's instructions, and reprobed with a ^{32}P-labeled 2.0 kb cDNA fragment of β-actin, provided by ClonTech.

Ribonuclease protection assay

Ribonuclease (RNase) protection assays were performed essentially as described (Pierceall *et al.*, 1994; Ekstrand *et al.*, 1995). A *NGN* riboprobe was generated from pAMP1-T07322, a plasmid containing a 312 bp *NGN* cDNA fragment (corresponding to amino acids 771–873). The *DCC* riboprobe has been previously described (Ekstrand *et al.*, 1995). To control for loading, γ-actin and β-actin riboprobes were used. The γ-actin riboprobe has been

described (Ekstrand *et al.*, 1995), and the β-actin riboprobe was prepared from the pTRI-β-actin-125-human plasmid construct (Ambion, Inc., Austin, TX). Probes were labeled with [^{32}P]CTP, and following purification through an acrylamide gel, 1.0×10^6 c.p.m. of the *NGN* and 2.0×10^5 c.p.m. of the γ-actin or β-actin riboprobe were incubated with 10 μg of RNA. Similarly, 1.0×10^6 c.p.m. of the *DCC* riboprobe and 2.0×10^5 c.p.m. of the γ-actin riboprobe were hybridized overnight with 20 μg of RNA. Non-hybridizing sequences were digested with RNase T2 (Gibco BRL Life Technologies). Protected fragments were recovered by ethanol precipitation and electrophoresed on a denaturing polyacrylamide sequencing gel. After drying the gel, autoradiography was carried out with X-OMAT film and intensifying screens.

RT–PCR assay

Total RNA was treated with two units of RNase-free DNase (Boehringer Mannheim). First-strand cDNA was prepared from three micrograms of RNA using AMV reverse transcriptase (Promega) and random hexamers. One tenth of the cDNA was used for each PCR with primer pairs derived from the human *DCC* and *NGN* sequences. Extracellular domain *NGN* primers (corresponding to amino acids 329–491) were NGN329S-5′-TTGAAGCTCAAGCAGAGCTTACAG-3′ and NGN-491A-5′-GACTGGTATTCTCAACACGTTCC-3′. *NGN* cytoplasmic domain primers (amino acids 1128–1239) were NGN1128S-5′-GTACCCGTCGTACCACCTCTCA-CC-3′ and NGN1239A-5′-CATCATTTTTGGTCTCATT-CCTCG-3′. *DCC* extracellular domain primers (amino acids 93–221) were DCC902S-5′-CAAATGGGTCTCT-GCTGATAC-3′ and DCCEX3A-5′-TCTTGAGCTGGC-TGGATTTCGAGC-3′. *DCC* cytoplasmic domain primers (amino acids 1110–1309) were DCK3090S-5′-CACAGT-GCTGGTAGTGGTCAT-3′ and DCK4504A- 5′-TTGGG-TTGATGGTCCTTCACTCAC-3′.

Amplifications were performed using the following conditions: hotstart followed by 35 cycles of 94°C × 45 s, 55°C × 45 s and 72°C × 2.5 min. One-fifth of each reaction was electrophoresed on 1.2% agarose gels and visualized with u.v. light following ethidium bromide staining. The identities of the *DCC* and *NGN* products were confirmed by Southern transfer and hybridization with their respective ^{32}P-labeled cDNA probes. A set of β-actin primers was used to independently confirm the first strand cDNA reaction. For all samples studied, the results with the two sets of *NGN* primers were concordant. Similarly, concordant results were obtained with the two sets of *DCC* primers.

Abbreviations

DCC - deleted in colorectal cancer; NGN - human neogenin; FN III - fibronectin type III, Ig - immunoglobulin; bp - base pair; kb - kilobase pair; RNase - ribonuclease; RT–PCR - reverse transcription polymerase chain reaction; VSV-G - vesicular stomatitis virus glycoprotein; kDa - kilodalton; ECL - enhanced chemiluminescence.

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Transcriptional Defects Underlie Loss of E-Cadherin Expression in Breast Cancer[1]

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Abstract

Decreased expression of E-cadherin (E-cad), a calcium-dependent cell adhesion molecule, has been seen in many different epithelial cancers. Although somatic mutations in the *E-cad* gene have been identified in a small subset of tumors, in the majority of cancers, the mechanisms underlying loss of *E-cad* expression are poorly understood. We have cloned the human *E-cad* promoter and defined its critical components in functional assays. In eight human breast cancer cell lines, there was a striking correlation between endogenous *E-cad* gene expression and the *E-cad* promoter activity observed following the introduction of reporter gene constructs into the lines. These and other observations suggest that defects in *trans*-acting pathways regulating *E-cad* expression are the primary basis for the loss of its expression in most breast cancers. The results have significant implications for understanding the gene expression differences that underlie tumor heterogeneity and progression events in breast and other epithelial cancers.

Introduction

E-cad,[3] a calcium-dependent transmembrane protein of roughly M_r 120,000, regulates epithelial cell-cell interactions at specialized regions of the plasma membrane called adherens junctions (1, 2). The function of E-cad depends critically upon its ability to link to the submembrane cytoskeletal matrix through its interactions with other proteins, such as α-, β-, and γ-catenin/plakoglobin (1–5). Alterations in the structure or expression of E-cad or the catenins have been found to promote aberrant cell-cell interactions *in vitro* (1, 2, 5, 6). Decreased or undetectable levels of E-cad expression have frequently been seen in immunohistochemical studies of many different epithelial cancers (reviewed in Ref. 2). In some cancers, loss of E-cad expression has been associated with the loss of differentiated features in tumor cells and/or increased propensity of the cells to invade and metastasize to distant sites. In addition, the restoration of E-cad expression following *E-cad* gene transfer has been shown to inhibit the invasive and metastic properties of the cells in *in vitro* and animal model systems (7–9)

The *E-cad* gene is located on chromosome 16q in a region that is frequently affected by allelic loss in several cancer types (10–13). Somatic mutations in the *E-cad* gene have been identified in more than 30% of gastric cancers of diffuse subtype, about 5–10% of endometrial and ovarian cancers, and about 5–10% of breast cancers, particularly those of lobular type (2, 14–17). The mutations identified include missense, nonsense, and splice mutations, as well as deletions. Nevertheless, in the majority of cancers in which *E-cad* expression is altered, the mechanisms accounting for its reduced or absent expression are poorly understood.

Two recent studies have presented apparently discordant conclusions on the mechanisms underlying loss of *E-cad* expression in cancer. A study by Graff *et al.* (18) concluded that *E-cad* expression was silenced in breast and prostate cancers by hypermethylation of the *E-cad* promoter sequences, whereas the findings of Hennig *et al.* (19) implied that the silencing of *E-cad* promoter activity in several different cancer types was due to loss of factor binding and/or chromatin rearrangement in the regulatory region. We report here the results of our studies to address the mechanisms underlying the loss of *E-cad* expression in breast cancer. Our findings of the human *E-cad* promoter suggest that defects in *trans*-acting pathways regulating *E-cad* gene expression are the primary mechanisms underlying loss of *E-cad* expression in breast cancer.

Results

Analysis of Human E-cad Promoter Activity. Previous studies have identified the transcription start sites for the murine and human *E-cad* genes (20, 21). In addition, several elements in the murine *E-cad* gene that regulate its expression in epithelial cells have been defined, including a 5′ promoter region, located within the 100-bp region immediately upstream of the transcription start site, and an enhancer region in the first intron (19, 20, 22, 23). Within the murine 5′ promoter region, a CCAAT-box and two candidate AP-2 binding sites in a GC-rich region have been characterized. In addition, a 12-bp palindromic element, located in the 5′ promoter region and called E-Pal, appears to be critical in directing epithelial-specific expression of *E-cad* (22, 23). Although the GC-rich region and CCAAT box are well con-

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[3] The abbreviations used are: E-cad, E-cadherin; AzaC, 5′-aza-2′deoxycytidine; Luc, luciferase; CMV, cytomegalovirus.

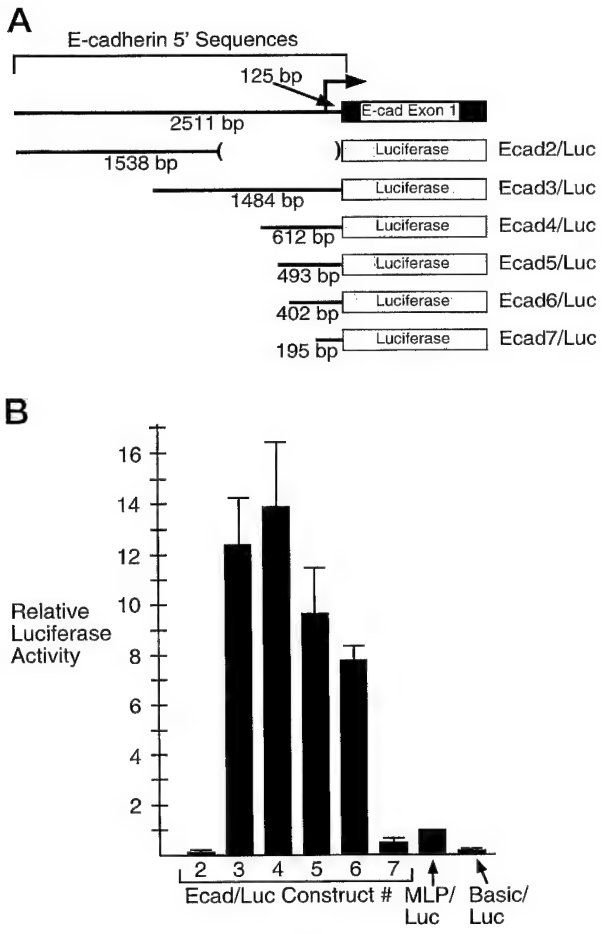

Fig. 1. Localization of promoter activity in human *E-cad* 5′ flanking sequences. *A*, for each *Luc* reporter gene construct, the extent of sequences 5′ to the *E-cad* initiating methionine is indicated. The proximal sequences deleted in the Ecad2/Luc construct are also indicated. The human *E-cad* transcriptional start site has been localized 125 bp upstream of the initiating methionine codon. In all reporter gene constructs, the *E-cad* initiating methionine codon has been destroyed, and a 28-bp flanking sequence separates the *E-cad* sequences from the initiating methionine of the *Luc* gene. *B*, relative Luc activity of the Ecad/Luc constructs and control constructs in MCF-7 breast cancer cells. Luc activities were determined by triplicate transfections of MCF-7 breast cancer cells with the indicated Luc constructs and a SV40-LacZ control construct. All Luc activities were normalized for β-galactosidase activity. The activity of an adenoviral late promoter Luc construct was assigned a value of one in each experiment. *Columns*, mean of the normalized Luc activities for all other constructs; *bars*, SD.

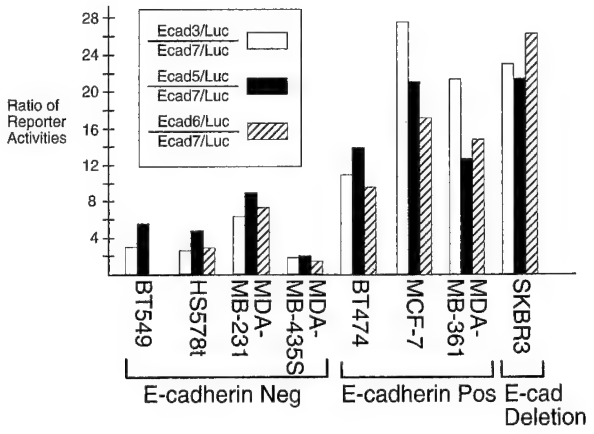

Fig. 2. Sequences between −70 and −277 in the human *E-cad* promoter are critical for its activity in breast cancer cell lines. The relative Luc activities of four *E-cad* Luc constructs were assessed in the eight breast cancer cell lines indicated. The Luc activities of the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs were compared to that of the Ecad7/Luc construct, and the mean ratio of the activities is indicated. The Luc activity of each construct was determined by three or more independent experiments, and all Luc activities were normalized for β-galactosidase activity. The *E-cad* expression status of the cell lines is indicated. The SKBR3 line lacks *E-cad* expression because of a homozygous deletion in the *E-cad* gene (16).

served in the human *E-cad* promoter region, the E-pal element is less well conserved (Ref. 21 and data not shown).

In an effort to further define the elements in the human *E-cad* promoter that are responsible for its transcriptional activity, we generated a panel of reporter gene constructs in which human *E-cad* 5′ flanking sequences of various extents were cloned upstream of the firefly *Luc* gene (Fig. 1*A*). We then characterized the Luc activities generated by these constructs following their transfection into MCF-7 and MDA-MB-361 cells, two breast cancer cell lines with high levels of endogenous *E-cad* expression. Similar activity profiles were obtained with the panel of constructs following transfection into each of the cell lines, although only the results for the MCF-7 cell line are shown in Fig. 1*B*. The constructs Ecad3/Luc, Ecad4/Luc, Ecad5/Luc, and Ecad6/Luc all generated Luc activities much greater than the reporter construct lacking any insert (*i.e.*, pGLBasic/Luc; Fig. 1*B*). The activities of most *E-cad* reporter constructs were even greater than the activity of a positive control vector containing the major late promoter of adenovirus (pMLP/Luc). The Ecad2/Luc vector lacks the *E-cad* transcription start site, and as expected, it failed to yield detectable levels of Luc following transfection (Fig. 1*B*). We also found that the E-cad7/Luc construct had weak activity. This observation implied that although the CCAAT and GC-rich elements present in the 70-bp region upstream of the *E-cad* transcription start site were sufficient for promoter activity, the elements conferred relatively weak activity compared to those present in constructs containing more 5′ *E-cad* sequences. We also noted that an *E-cad* reporter construct containing roughly 2.5 kb of *E-cad* 5′ flanking sequences had very reduced promoter activity (data not shown), suggesting that inhibitory elements may be present upstream of the more proximal E-cad promoter elements.

Activity of E-cad Promoter Constructs Parallels Endogenous Gene Activity. Studies were undertaken with a subset of the Ecad/Luc constructs in a panel of eight breast cancer cell lines to further assess the relationship between reporter gene activity and endogenous *E-cad* expression in the lines. As noted above, the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs had considerably more activity than the E-cad7/Luc construct in MCF-7 cells and MDA-MB-361 cells. As shown in Fig. 2, these findings were confirmed and extended to an additional breast cancer cell line with moderate to high levels of endogenous E-cad expression (BT474; Ref. 16). However, in four breast cancer lines lacking endog-

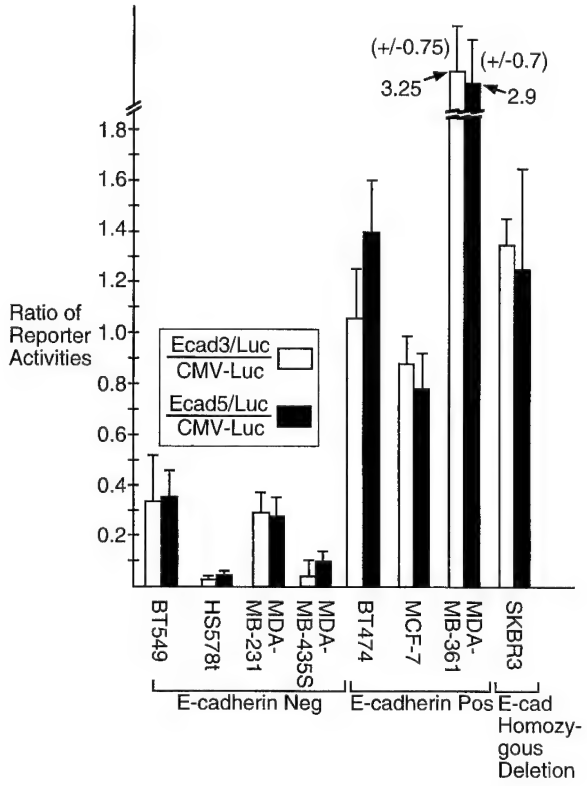

Fig. 3. E-cad promoter activity is correlated with endogenous *E-cad* activity in breast cancer cell lines. The relative Luc activity of two different *E-cad* Luc constructs (Ecad3/Luc and Ecad5/Luc; see Fig. 1) in eight breast cancer cell lines is indicated. The Luc activities of the two *E-cad* reporter constructs were compared to the Luc activity of a control CMV-driven reporter construct (pUHC-13-3). Luc activities in each cell line were determined by three to five independent experiments with the *E-cad* and CMV-Luc constructs and a SV40-LacZ control construct. All Luc activities were normalized for β-galactosidase activity. *Columns,* mean ratio of the luciferase activities; *bars,* SD.

enous *E-cad* expression and without evidence for mutational inactivation of the *E-cad* gene (BT549, HS578t, MDA-MB-231, and MDA-MB-435S; Ref. 16), the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc vectors had considerably less activity (Fig. 2).

SKBR3 cells lack *E-cad* expression because a substantial portion of the *E-cad* gene is affected by homozygous deletion (16). Given that the homozygous deletion in SKBR3 is clearly sufficient for complete inactivation of *E-cad*, the pathways and transacting factors regulating *E-cad* promoter activity might be expected to be intact in SKBR3. Consistent with this prediction, following transfection into the SKBR3 cell line, we found that the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs all had considerably greater activity than the Ecad7/Luc construct (Fig. 2). Additional studies comparing the activities of the Ecad3/Luc and Ecad5/Luc constructs with that of a control CMV-Luc vector further established that the *E-cad* promoter activities closely paralleled endogenous E-cad activity in the eight cell lines (Fig. 3). *In toto*, our studies indicate that *trans*-acting factors that interact with sequences between 70 and 277 bp upstream of the E-cad transcriptional start site are likely to be critical in regulating E-cad expression in breast epithelial cells. Defects in the expression or regulation of one or more of these transacting factors appear to be a major contributor to the loss of E-cad expression in a number of breast cancer cell lines.

AzaC Treatment Fails to Reactivate E-cad Expression. As discussed above, the previous studies of Graff *et al.* (18) had shown that hypermethylation of the *E-cad* proximal promoter region was correlated with decreased *E-cad* expression in a panel of breast and prostate cancer cell lines. In addition, these authors reported that treatment of selected breast and prostate cancer cell lines with the demethylating agent AzaC reactivated *E-cad* expression. In particular, using Western blot and immunofluorescence studies, the authors found minimal reactivation of *E-cad* expression in the MDA-MB-231 and HS578t breast cancer cell lines following exposure of the cells to 0.5 μM AzaC for 3 days (18).

We sought to assess E-cad expression by Western blot analysis in five *E-cad*-negative breast cancer cell lines following exposure of the cells to various levels of AzaC. All five lines lacked detectable *E-cad* mutations (16). As shown in Fig. 4, we failed to detect E-cad expression by Western blot analysis in any of the *E-cad*-negative breast cancer cell lines treated with 1 or 3 μM AzaC for 5 days, including the two breast lines (*i.e.*, MDA-MB231 and HS578t) studied by Graff *et al.* Two factors complicate definitive interpretation of the effects of AzaC treatment on endogenous E-cad expression. First, our Western blot analysis may have been somewhat less sensitive than the Western blot and immunofluorescence studies of Graff *et al.* (18). Second, similar to the findings of Graff *et al.* (18), we found that AzaC treatment had essentially no detectable effects on the methylation status of the proximal *E-cad* promoter in the breast cancer cell lines (data not shown). Although these caveats should be borne in mind, our results clearly demonstrate that E-cad expression cannot be reactivated to the levels seen in *E-cad*-positive breast cancer cell lines by brief AzaC treatment (Fig. 4).

Factors Regulating E-cad Promoter Activity. Although our transfection studies with unmethylated report gene constructs indicate that *trans*-acting defects are likely to be the predominant mechanism underlying the loss of *E-cad* promoter activity in breast cancers, we sought to explore the possibility that methylation of CpG dinucleotide sites in the *E-cad* promoter region might cooperate with transcriptional defects to further extinguish *E-cad* expression. We compared the Luc activities of an unmethylated *E-cad* reporter gene vector to the Luc activities generated by the vector following its *in vitro* methylation with purified bacterial *Hha*I methylase or *Hpa*II methylase. High levels of Luc activity were generated by the unmethylated Ecad5/Luc vector in the *E-cad*-positive MDA-MB-361 cell line, whereas *in vitro* methylation of the vector with *Hha*I or *Hpa*II methylase markedly decreased Luc activity (Fig. 5). In the *E-cad*-negative cell line MDA-MB-435S, no significant effects on promoter activity were seen when the unmethylated Ecad5/Luc reporter construct was compared to the same vector methylated *in vitro* with *Hha*I or *Hpa*II methylase.

Hennig *et al.* (19, 23) have provided evidence that AP-2 or an AP-2-related factor binds to two tandem sites in the

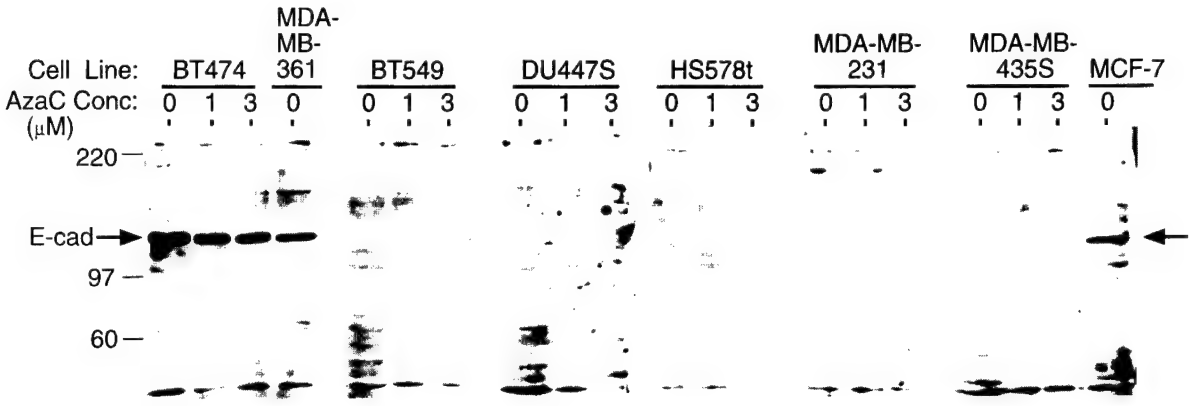

Fig. 4. E-cad expression in breast cancer cell lines is not reactivated by treatment with AzaC. Western blot analysis was carried out to assess E-cad expression in cell lines following a 5-day treatment with AzaC at 0, 1, or 3 μM. *Top,* identity of the cell lines; *arrow,* relative mobility of E-cad; *left,* molecular weight markers (in thousands). The cell lines BT474, MDA-MB-361, and MCF-7 are *E-cad*-positive, and the other five cell lines are *E-cad*-negative.

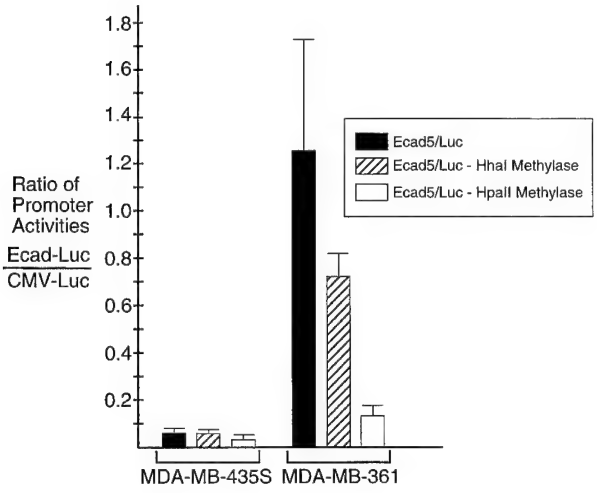

Fig. 5. Methylation of the *E-cad* promoter inhibits its activity. The relative Luc activity of the *Ecad5/Luc* reporter gene construct was assessed in the MDA-MB-435S and MDA-MB-361 cell lines following *in vitro* methylation with either *Hha*I or *Hpa*II methylase. Because transfection efficiencies differed among the lines, the Luc activity generated by the Ecad5/Luc construct was compared to the Luc activity of a control CMV-Luc reporter construct (pUHC-13-3). Luc activities were determined by three independent experiments, and all Luc activities were normalized for β-galactosidase activity. *Columns,* mean ratio of the luciferase activities; *bars,* SD.

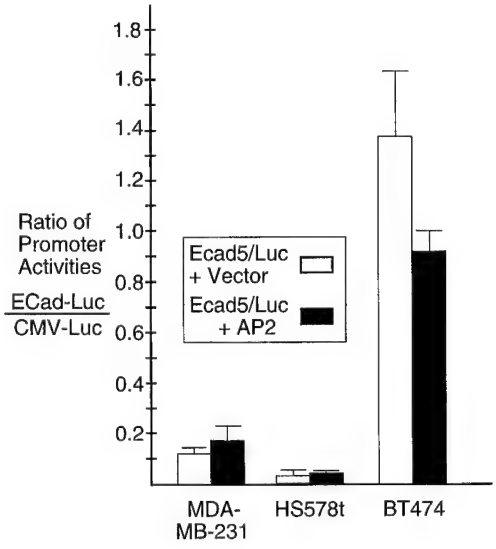

Fig. 6. E-cad promoter activity is not activated by AP-2 transfection. Shown in the figure are the relative Luc activities generated following cotransfection of three different breast cancer cell lines with the *Ecad5/Luc* reporter gene construct and either a pcDNA3 expression vector with an AP2 cDNA or the empty expression vector. Because transfection efficiencies varied among the cell lines, the Luc activities generated by the Ecad5/Luc construct were compared to the Luc activity of a control CMV-Luc reporter construct (pUHC-13-3). Luc activities were determined by three independent experiments, and all Luc activities were normalized for β-galactosidase activity. *Columns,* mean ratio of the luciferase activities; *bars,* SD.

GC-rich region of the murine *E-cad* promoter and that this region is critical in the regulation of *E-cad* expression, although their findings also suggest that the GC-rich region functions together with the CCAAT-box and E-pal elements to confer tissue-specific expression of E-cad. On the basis of the studies of AP-2 expression in breast cancer cell lines carried out thus far (24), there is little evidence for a correlation between *E-cad* expression and AP-2 expression, although only a limited number of cell lines have been studied. Nevertheless, to more directly assess the role of AP-2 in regulating *E-cad* expression in breast cancer, we transfected an AP-2 cDNA together with the Ecad5/Luc vector into two *E-cad*-negative cell lines. The AP-2 cDNA failed to activate the E-cad promoter in either the MDA-MB-231 or HS578t cell lines (Fig. 6). A small, but reproducible, decrease in *E-cad* promoter activity was seen in the BT474 E-cad-positive cell line following transfection of the AP-2 cDNA. The findings imply that loss of AP-2 activity is not likely to underlie the frequent loss of *E-cad* expression in breast cancer.

Discussion

A sizable fraction of breast cancers have been found to have reduced or absent *E-cad* expression, although mutations in the *E-cad* gene have only been identified in a very small

subset of the *E-cad*-negative cancers (2, 16, 17). In an effort to further understand the mechanisms underlying loss of *E-cad* expression, we have cloned the human *E-cad* promoter and have undertaken sequence and functional analysis of its elements in breast cancer cells. We found that the Ecad7/Luc reporter construct, although it contains the proximal 70 bp of *E-cad* sequence upstream of the transcription start site and the previously defined *E-cad* regulatory elements (*i.e.*, CCAAT box, two candidate AP-2 binding sites, and E-pal element), had only weak promoter activity. In contrast to the weak activity seen with the Ecad7/Luc construct, the Ecad6/Luc construct, containing a 275-bp fragment of *E-cad* proximal promoter sequences, had nearly the promoter activity of constructs containing considerably larger *E-cad* 5′ fragments of 500–1350 bp (*e.g.*, Ecad3/Luc and Ecad5/Luc). Thus, the findings imply that critical regulatory elements in the human *E-cad* promoter reside in the approximately 200-bp region immediately upstream of the previously defined minimal promoter elements. Furthermore, we noted a striking relationship between the endogenous *E-cad* gene activity in breast cancer cell lines and the promoter activities of the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs when they were introduced into the cells. Our studies strongly suggest that defects in signaling pathways or *trans*-acting transcription factors that regulate *E-cad* expression are likely to be the primary mechanisms underlying the loss of *E-cad* expression in breast cancers.

Our results are consistent with those of two previous studies in which murine *E-cad* promoter activity was correlated with endogenous *E-cad* expression in several human cancer cell lines, including a total of four breast cancer lines (19, 22). Studies of the activity of a minimal human *E-cad* promoter in two prostate cancer cell lines have also suggested a correlation between endogenous *E-cad* expression and activation of *E-cad* promoter elements (21), although we have not found as clear-cut a relationship in our preliminary studies in prostate cancer cell lines.[4] Consistent with the results of the promoter activity studies, *in vivo* footprinting analyses have demonstrated protection of several distinct elements in the minimal *E-cad* promoter in *E-cad*-positive cancer cell lines but not in *E-cad*-negative lines (19).

Others have suggested that methylation of the *E-cad* promoter region may be responsible for the loss of *E-cad* expression in cancer, and increased methylation of the *E-cad* proximal promoter region has been identified in cancers and cancer cell lines lacking E-cad expression (18), an observation that we also confirmed in the lines studied here (data not shown). In a previous study, treatment of selected breast and prostate cancer cell lines with the demethylating agent AzaC was reported to reactivate *E-cad* expression in a minimal fraction of the treated cells (18). However, we failed to detect E-cad protein expression in any of five *E-cad*-negative breast cancer cell lines treated with AzaC, demonstrating that *E-cad* expression cannot be reactivated to the levels seen in *E-cad*-positive breast cancer cell lines by brief AzaC treatment. By studying *E-cad* promoter constructs in which CpG dinucleotides had been methylated *in vitro* by purified methylases, we did obtain support for the proposal that methylation of CpG dinucleotide sites in the *E-cad* promoter region might cooperate with transcriptional defects to further extinguish *E-cad* expression.

The binding of AP-2 or an AP-2-related factor to two tandem sites in the GC-rich region of the murine and human *E-cad* promoter has been suggested to regulate *E-cad* expression (23). However, we found that transfection of an AP-2 cDNA failed to activate the *E-cad* promoter in either of two *E-cad*-negative cell lines. Hence, loss of AP-2 is not likely to underlie the frequent loss of *E-cad* expression in breast cancer. Others have suggested that overexpression of HER-2/neu may cause a decrease in *E-cad* expression (25), although we failed to demonstrate a correlation between *E-cad* and HER-2/neu expression in breast cancer in our previous studies (16). Therefore, although the present evidence supports the proposal that defects in signaling and/or transcription factor pathways are the predominant mechanisms underlying altered *E-cad* expression in breast cancer, additional studies are clearly needed to elucidate the specific nature of these defects. Further characterization of these defects should provide new and important insights into the pathogenesis of breast cancer and the mechanisms underlying tumor cell heterogeneity and progression.

Materials and Methods

Plasmid Constructs. Genomic clones containing human *E-cad* exons 1 and 2, as well as 5′ flanking sequences, were isolated from a human genomic DNA library generously provided by Dr. Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, MD) using a human *E-cad* cDNA probe and multiple rounds of hybridization selection. An approximately 2.5-kb *Sal*I-*Nco*I fragment extending 5′ from the initiating methionine codon in exon 1 was identified and subcloned into pBluescriptII (Stratagene, La Jolla, CA). Deletions of varying extent in the *E-cad* sequences were generated using exonuclease III and mung bean nuclease (Stratagene). A series of *Luc* reporter gene constructs containing *E-cad* 5′ flanking sequences of various extents was generated by subcloning the *E-cad* sequences into the *Sac*I and *Hin*dIII sites of the pGL2-Basic vector (Promega Corp., Madison, WI) immediately upstream of the coding region of the firefly *Luc* gene. During the subcloning of each *E-cad* fragment, the *E-cad* initiating methione codon was destroyed. The identities of the *E-cad* sequences present in the vectors were confirmed by sequence analysis. The control Luc vectors pUHC-13-3 and pMLP/Luc, containing CMV and adenovirus major late promoter elements, respectively, have been described previously (26, 27). The pCH110 plasmid, containing a functional *LacZ* gene expressed under control of the SV40 early promoter, was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). The vector pcDNA3-AP2 was constructed by subcloning a 1.6-kb *Hin*dIII-*Eco*RI murine AP-2 cDNA fragment (kindly provided by Dr. Trevor Williams, Yale University) into the *Hin*dIII and *Eco*RI sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). All plasmid DNAs were isolated using reagents from QIAGEN, Inc. (Chatsworth, CA). *In vitro* methylation of the plasmid vector pEcad5/Luc was carried out on 20 μg of DNA using purified *Hha*I or *Hpa*II methylase (New England Biolabs, Inc., Beverly, MA) and the manufacturer's recommended reaction conditions.

Cell Culture. All cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in the recommended growth media. Cultures were incubated at 37°C with 5% CO_2 except for those maintained in Lebovitz's L-15 medium, which were grown at 37°C without CO_2. Selected cell lines were treated with 1 or 3 μM AzaC (Sigma Chemical Co., St. Louis, MO) for 5 days.

Transfections and Luc and β-Galactosidase Reporter Assays. Cell lines growing at roughly 70% confluence were transfected in six-well plates using 1 ml of Opti-MEM reduced serum medium (Life Technologies,

[4] X. Ji, E. R. Fearon, and R. Morton, unpublished observations.

Inc., Grand Island, NY), 4 μl of Lipofectin reagent (Life Technologies, Inc.), 0.8 μg of the indicated pGL2 or the pUHC-13-3 control Luc plasmid, and 0.8 μg of pCH110. Transfections to assess the affect of AP-2 were undertaken as described above, except that 8 μl of Lipofectin and 0.8 μg of pEcad5/Luc, 0.8 μg of pCH110, and 0.8 μg of pcDNA3-AP2 or pcDNA3 plasmid without a cDNA insert were used. Cell extracts were prepared 36–40 h posttransfection using reporter lysis buffer (Promega). One-fifth and one-half of the lysate were used for the Luc and β-galactosidase assays, respectively. Both assays were carried out as recommended by the manufacturer (Promega). Luc activities were measured in a luminometer (model TD-20E, Turner Corp., Mountain View, CA). All transfections were repeated three or more times.

Western Blot Analysis. Lysates were prepared in radioimmunoprecipitation assay buffer with protease inhibitors, as described previously (16). Approximately 40 μg of total protein per sample was separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA) by semi-dry electroblotting (Transblot, Bio-Rad, Hercules, CA). Western blot analysis of E-cad was carried out using the DECMA-1 rat monoclonal antibody (Sigma) and a horseradish peroxidase-conjugated goat antirat antibody (Pierce). AP-2 was detected with the AP-2 (C18) affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a horseradish peroxidase-conjugated goat antirabbit secondary antibody (Pierce). Antibody complexes were detected with the ECL Western kit blot (Amersham Corp., Arlington Heights, IL) and exposure to X-OMAT film.

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Localization and Quantitation of Expression of the Cell Motility–Related Protein Thymosin β15 in Human Breast Tissue

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Thymosin β15 is a newly discovered 5300-Da protein that binds actin monomers and inhibits actin polymerization and might thus increase cellular motility. Thymosin β15 is upregulated at both the mRNA and protein levels in prostate cell lines in a manner directly related to their capacity to metastasize. We hypothesize that because this protein is upregulated in cells with a propensity to metastasize, it might be a useful prognostic marker in breast cancer. Because this is a newly described protein, neither the subcellular localization of thymosin β15 or its expression in breast cancer has been examined. We describe the use of an affinity-purified polyclonal antibody to show that within breast epithelium, thymosin β15 is localized diffusely throughout the cytoplasm and that thymosin β15 is upregulated in malignant (compared with benign) breast tissue. In contrast to the prostate model, thymosin β15 is upregulated in nonmetastatic breast cancer and even ductal carcinoma *in situ* (compared with benign breast tissue), and, consequently, it might represent a potential early marker for breast malignancy. Additional studies are needed to evaluate the precise role and prognostic value of thymosin β15 in breast cancer.

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β thymosins are a family of closely related, highly polar, 5-kDa polypeptides. All of the vertebrates studied and some invertebrates are known to contain one or often two β thymosins (1). Thymosin β15 was recently uncovered in a search for proteins with increased expression in motile, as compared with poorly motile, Dunning rat prostatic carcinoma cell lines (2). The protein, which is 5300 Da, was designated "thymosin β15" because of its approximately 60% homology with other members of the β thymosin family.

Thymosin β4 is the most abundant β thymosin in most mammalian tissue and is the best studied member of this family. Current understanding is that thymosin β4 sequesters a large pool of monomeric actin that is accessible to be released as needed for polymerization of actin filaments (3–5). Microinjection or overexpression of thymosin β4 causes disassembly of actin stress fibers (6–8). Like thymosin β4, thymosin β15 binds monomeric actin and inhibits actin polymerization, abilities that confirm its place in the β thymosin family. Thymosin β15 can also positively regulate cell motility, because transfection of antisense thymosin β15 into motile rat prostatic carcinoma lines impairs cell motility in a Boyden chamber apparatus (2).

The putative role of β thymosins in modulation of the actin cytoskeleton through monomer sequestration suggests that they might be involved in cell differentiation, carcinogenesis, and metastasis. In some but not all cell lines, increased thymosin β4 protein or mRNA correlated with differentiation (9). In human tumors, thymosin β4 mRNA increased in hairy cell leukemia and decreased in some lymphomas (10). Two of three metastatic colorectal carcinomas showed decreased thymosin β4 mRNA, compared with nonmetastatic tumors, with the

third metastatic tumor showing little change (11). Thymosin β10 mRNA levels increased in renal cell carcinomas (12, 13), and thymosin β10 upregulation correlated with the metastatic potential of melanomas (14). The expression of each thymosin β family member is independently regulated. Consequently, different family members might be independently increased or decreased in particular tumor types.

Because thymosin β15 has only recently been described, it is less well characterized. It was shown to be upregulated in metastatic human prostate cancers at both the mRNA and protein level when compared with less metastatic prostate cancers (2). Immunostaining of human prostate cancers revealed a general correlation between Gleason grade and thymosin β15 expression, with high-grade tumors (Gleason Grade 8–10) showing more staining than did low-grade tumors (Gleason Grade 2–5).

The promising preliminary data from the prostate studies prompted us to undertake a study of thymosin β15 expression in human breast tissue. Our aim was to assess the tissue and cellular localization of thymosin β15 and to evaluate whether thymosin β15 expression is associated with malignant changes of the breast epithelium. To do so, breast tissue samples representing a range of conditions from normal to neoplastic were examined using affinity-purified polyclonal anti-thymosin β15 antibodies. We describe a significant association of high levels of expression of this protein with breast malignancy.

MATERIALS AND METHODS

Patients and Tumor Specimens

Forty-two formalin-fixed, paraffin-embedded tissue blocks were obtained from patients who underwent breast biopsies or breast resections between 1989 and 1996 at Yale–New Haven Hospital, New Haven, Connecticut. Twenty-one blocks of specimens from breast biopsies that yielded a benign primary diagnosis were selected, and an additional 21 blocks from resections or biopsies that yielded a malignant diagnosis of either ductal carcinoma *in situ* (DCIS), infiltrating ductal carcinoma, or infiltrating lobular carcinoma were selected. Clinical information corresponding to the 42 blocks was obtained from the Yale–New Haven Hospital pathology database.

Antibody

An affinity-purified polyclonal antibody raised against the ^{11}C-terminal amino acids of thymosin β15 was used. The preparation and purification of this antibody was previously described (2).

Immunohistochemical Analysis

Standard histologic sections were cut from the paraffin blocks, baked overnight at 60° C, and deparaffinized. Slides were then soaked in 0.75% hydrogen peroxide in methanol to quench endogenous peroxidases. For antigen retrieval, each slide was immersed for 5 minutes in 6.5 mM sodium citrate, pH 6.0, in a heated conventional pressure cooker (15). After a 1-hour incubation in 0.3% bovine serum albumin diluted in triethanolamine-buffered saline (TBS), pH 8.0 (Sigma, St. Louis, MO), for the slides stained by immunofluorescence or normal serum from the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) diluted in TBS, pH 8.0, to block nonspecific binding, the slides were incubated for 1 hour with the primary anti–thymosin β15 antibody, diluted 1:100 in the solution used for blocking. The slides were then washed seven times. The slides were then incubated for 1 hour with secondary antibody. Cy3-conjugated goat anti–rabbit immunoglobulin G (Jackson ImmunoResearch Labs, West Grove, PA), diluted 1:500 in TBS, pH 8.0, was used as the secondary antibody for the immunofluorescent specimens, and biotinylated goat/anti–rabbit immunoglobulin G, diluted 1:200 in TBS, pH 8.0, was used for the peroxidase-stained specimens. The slides were then washed seven times. The peroxidase-stained slides were incubated 30 minutes in the Vectastain ABC reagent (Vector) prepared in TBS, pH 8.0, which is composed of avidin and biotinylated horseradish peroxidase, and then washed for 5 minutes in TBS, pH 8.0. All of the incubations were accomplished by coating the tissue with solution and storing the slides in a humidity chamber to prevent evaporation. The peroxidase-stained slides were then developed using the Vector 3,3′-diaminobenzidine kit (Vector) for 6 minutes, which uses diaminobenzidine and hydrogen peroxide. The peroxidase-stained slides were counterstained with hematoxylin. The immunofluorescent slides were coverslipped with *N*-propyl gallate and sealed with nail polish, and the peroxidase-stained slides were coverslipped with Immuno-mount (Shandon, Pittsburgh, PA). The immunofluorescent slides were stored at −20° C to preserve the signal, and the peroxidase-stained slides were stored at room temperature. Negative controls were prepared by substituting the primary antibody with blocking solution in each protocol.

Histologic Scoring and Analysis

The immunofluorescent slides were examined by two individuals (JSG, DLR) on an Olympus AX-70 (XXX, XX) epifluorescence photomicroscope, and representative photographs were obtained. For the peroxidase-stained specimens, each slide was ex-

amined by three observers (JSG, RADG, DLR), using a standard light microscope. For each slide, each observer assigned up to five scores representative of different pathologic elements contained on that slide. Each slide was scored for the staining of normal glands, hyperplastic glands, all benign cells, DCIS, and infiltrating cancer. The scores were based on the average staining of all the cells representing that diagnosis on the slide. Cells were scored as 0 if the staining was no greater than background, 1+ if staining was slightly greater than background, 2+ for an intermediate level of staining, and 3+ for very intense staining. If the observer deemed there to be insufficient or no tissue representing a particular diagnosis, a score was not assigned for that slide. On the basis of the scores from the three observers, a summary score was assigned for each element on each slide by totaling the scores if all three observers assigned scores. If only two observers assigned scores, the sum was multiplied by 3/2 to bring the summary score to the correct scale. A summary score was not given if one or no observers scored a particular diagnosis on a slide. Data analysis was accomplished using StatView 4.5 for Macintosh (XXX, XXX, XX).

A total score (rather than a consensus score) was computed for each case to reflect the input of the three observers. The fact that the scores largely agreed lent validity to the study. In no case was there major discrepancy in scoring, *e.g.*, a score of 0 by a one reviewer and 3+ by another. Only two (3%) of the cases had two-point discrepancies (1+ *vs.* 3+). The remaining 97% of the cases either were scored identically by all of the three reviewers or had a one-point discrepancy, *e.g.*, two reviewers gave the case a 3+, whereas the third scored it as a 2+). Given this high degree of consensus, we were tempted simply to assign consensus scores, but we decided that preserving all of the scores gave a more accurate picture of the distribution of the staining pattern.

Optimally, we could score each case as positive or negative for expression, but because the level of expression is scored subjectively, we used a continuous scoring system. The rationale for using the cutoff of five to separate the positive from the negative cases is twofold. The first rationale for this division is theoretical. Because three-point discrepancies never occurred and two-point discrepancies were very rare, a case had to be scored as having at least intermediate staining by at least two reviewers to obtain a score of five. This seemed like a reasonable criterion to consider a case as positive. The second rationale for this division is that the score of five would be extremely sensitive for a diagnosis of malignancy, with 94% of the cases scoring five or higher and only one case scoring below five. Also, the peak of the distribution for the benign cases lies below five, with 65% of the cases scoring less than five. Ultimately, we found that using five as a cutoff does indeed provide the best separation between the benign and malignant cases.

RESULTS

Location

Paraffin-embedded tissue from both benign and malignant breast lesions was stained by the immunofluorescence method to evaluate the subcellular localization of thymosin β15 and the relative specificity of the affinity-purified anti–thymosin β15 antibody. In all of the breast epithelial cells, staining for thymosin β15 was located diffusely throughout the cytoplasm and was excluded from the nucleus (Fig. 1). The intensity of the staining of breast epithelial cells varied from slide to slide and even within the same slide. Although the intensity of the staining was not formally scored in the slides stained by the immunofluorescence method, it was clear that there was at least one example for both benign and malignant glands where there was intense staining and at least one example for each of staining that was not significantly greater than background. The anti–thymosin β15 antibody stained occasional endothelial cells in addition to the breast epithelial cells. Also, in some slides, it seemed to stain more intensely in myoepithelial cells (Fig. 1), but this finding was not represented in all of the specimens.

Expression

For ease of interpretation, the remainder of study was done using conventional peroxidase-based staining and a standard light microscope. Forty-three slides of surgical specimens representing a spectrum of breast lesions were stained using the anti–thymosin β15 antibody and visualized with a biotinylated secondary antibody, peroxidase-linked avidin, and diaminobenzidine substrate. Twenty-one slides had primary diagnoses that represent benign changes, and 22 slides had a primary diagnosis of malignancy (6 DCIS, 16 infiltrating cancers). Many slides contained tissue representing more than one diagnosis, but there were no malignant changes on any of the slides for which the final diagnosis was benign and no infiltrating cancer on slides from cases with the final diagnosis of DCIS. All of the slides were from female patients, and the average age of the patients was 43 years for the benign slides and 59 years for the malignant slides.

As with the immunofluorescent slides, there was some minimal background staining of the stroma. There were variable levels of thymosin β15 expression between the slides, such that the level of stain-

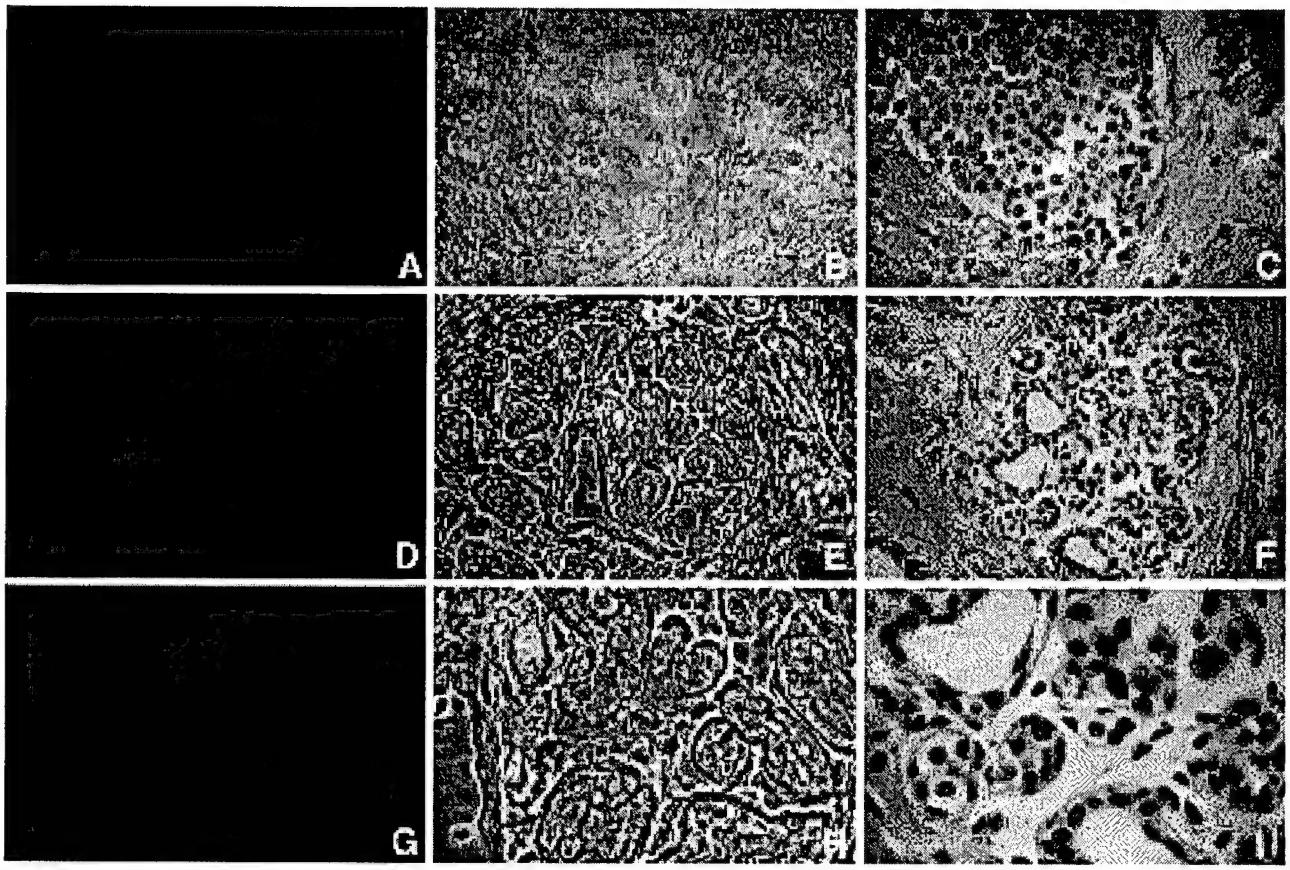

FIGURE 1. Expression of thymosin β15 in breast tissue. **A,** the expression pattern of thymosin β15 in infiltrating ductal carcinoma is visualized by staining with an affinity-purified polyclonal antibody directed against thymosin β15 followed by a Cy3 conjugated goat anti-rabbit secondary antibody. Thymosin β15 is localized diffusely throughout the cytoplasm in the tumor cells but is excluded from the nucleus. **B,** the corresponding phase image of the same field. **C,** the same structure in a serial section stained with hematoxylin and eosin. **D,** an immunofluorescent image of the myoepithelial cell staining pattern for thymosin β15. **E,** corresponding phase. **F,** hematoxylin and eosin stain. Original magnification, 400× for **A-F,** 1000× for **G-I.**

ing could be scored with relation to background. Each of the slides was examined and scored by three people. Examples of the different scores are shown in Figure 2. The three individual scores were compiled to give combined scores on a scale from 0 to 9. Table 1 shows a summary of the benign cases, including the diagnosis and overall score. Table 2 contains similar data for the malignant cases, but, in addition to the overall score of the benign elements, it also includes scores for the DCIS and invasive cancer regions on each slide. For the slides with infiltrating cancer, the status of the axillary lymph nodes with respect to metastases, the size of the resected tumor, the estrogen and progesterone receptor status, and the ploidy of the tumor are shown.

A summary of all of the data separated by diagnosis is provided in Table 3. The average score shown represents the score for the combined elements for those slides with a benign primary diagnosis, for the DCIS on the DCIS slides, and for the infiltrating cancer on the slides with infiltrating cancer. The score for each slide was converted to a binary value of either positive (scores ≥ 5) or negative (scores < 5). The percentage of slides in each diagnosis scored as positive is also shown in Table 3. The distribution of the scores by tissue type is shown in Figure 3, illustrating the preponderance of stronger staining in malignant epithelium than in benign epithelium and justifying the division point for the binary scoring.

To evaluate these data for significance, a contingency table was constructed (Table 4). Calculation of the χ^2 statistic shows a statistically significant increase in the number of malignant slides scored positive, compared with the benign slides (P = .0002). There were also more DCIS slides scored as positive (P = .0149) and infiltrating cancer slides scored as positive (P = .0014) when these were compared individually to the benign slides. The slides of tumors with and without nodal metastasis were both more positive than the benign slides when compared individually (P = .0329 and P =.0311, respectively). Among the selected slides, there were nine cases that allowed the comparison between malignant cells and benign cells from the same patient (on the same slide). In this group, the malignant cells received more positive scores than the adjacent benign cells (P = .0034). No correlation was found in staining pattern with age, node

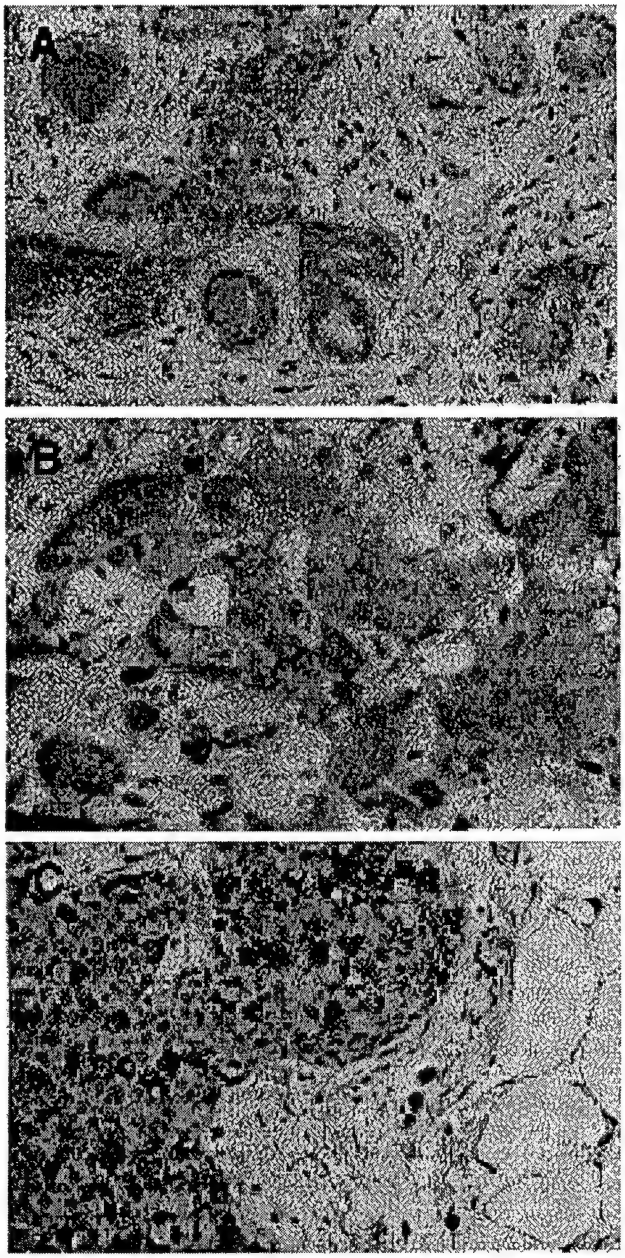

FIGURE 2. Variation in the intensity of staining of thymosin β15 in breast epithelial cells. Histologic sections were stained with an affinity-purified polyclonal antibody directed against thymosin β15 and visualized with a peroxidase/3,3′-diaminobenzidine method. **A,** expression of thymosin β15 in benign breast ducts that is slightly greater than background. **B,** an intermediate expression level as seen in an infiltrating ductal carcinoma. **C,** a very intense staining pattern is seen in another case of infiltrating ductal carcinoma. These figures illustrate the scoring system defined for thymosin β15 expression in breast epithelial cells. The level of thymosin β15 expression was scored as either 1+ (A), 2+ (B), or 3+ (C). Negative controls with goat serum only were used as a baseline reference for each case.

TABLE 1. Summary of Clinical Data and Thymosin β15 Staining of Benign Cases

Primary diagnosis	Age (yr)	Score
Fibroadenoma	36	6.0
	34	4.0
	24	4.0
	58	3.0
	18	7.0
	25	4.0
	37	2.0
	27	8.0
	60	3.0
Fibrocystic disease	40	3.0
Sclerosing adenosis	53	3.0
	56	6.0
	42	5.0
	43	3.0
	60	4.0
Atypical hyperplasia	54	7.0
	44	5.0
	29	—
	46	3.0
	57	4.0
	63	3.0

status, size, estrogen receptor status, progesterone receptor status, or ploidy.

DISCUSSION

Thymosin β15, like other members of the β thymosin family, is known to bind actin monomers and is thought to be associated with cell motility. Evidence that it is upregulated in human prostate cancers and that upregulation seems to correlate with disease severity suggests that it might mark metastatic potential (2). This study suggests that breast tissue might show similar properties, *i.e.*, upregulation is associated with malignant changes of the ductal epithelium. Although the definition of progressive stages of severity in breast cancer is less well quantitated than it is in prostatic cancer, both DCISs and infiltrating ductal carcinomas contain more thymosin β15 than does benign breast tissue. One measure of severity or progression in breast cancer is metastasis. In this study, seven of eight tumors with nodal metastases at presentation showed positive staining, consistent with upregulation of this protein in association with motility. Ours is a pilot study, which has too few samples for a quantitative assessment of this trend.

Although less well defined than progression in colorectal carcinoma, it is generally presumed that there is a progression from DCIS to nonmetastatic infiltrating ductal carcinoma to metastatic breast cancer. Both DCIS and node-negative breast cancer have increased thymosin β15 expression in this study. Thus, it seems that upregulation of thymosin β15 could occur early in the oncogenic pathway.

In this study, some malignant lesions stained weakly or moderately, whereas some of the benign tissue seemed to have a strong staining pattern. We are unsure of the significance of this observation. It is conceivable that there is a wide variation in thymosin β15 expression within a given lesion. Although there are examples of benign tissue with increased expression and malignant tissue with baseline expression, the overall trend of malig-

TABLE 2. Summary of Clinical Data and Thymosin β15 Staining of Malignant Cases

Primary diagnosis	Age (yr)	Node status	Tumor size	ER status	PR status	Ploidy	Benign score	DCIS score	Invasive cancer score
DCIS	53	N/A	N/A	N/A	N/A	N/A	4.0	8.0	N/A
	25	N/A	N/A	N/A	N/A	N/A	3.0	5.0	N/A
	46	N/A	N/A	N/A	N/A	N/A	5.0	—	N/A
	83	N/A	N/A	N/A	N/A	N/A	4.5	6.0	N/A
	41	N/A	N/A	N/A	N/A	N/A	3.0	5.0	N/A
	65	N/A	N/A	N/A	N/A	N/A	4.0	7.0	N/A
Ductal carcinoma	69[a]	Positive	3.0	Negative	Negative	Diploid	—	—	9.0
	69[a]	Positive	3.0	Negative	Negative	Diploid	3.0	—	—
	45	Positive	10.0	Negative	Negative	Unknown	3.0	7.0	7.0
	44	Positive	2.5	Unknown	Unknown	Unknown	—	7.0	6.5
	38	Positive	Unknown	Positive	Negative	Aneuploid	3.0	—	3.0
	64	Positive	4.5	Negative	Negative	Unknown	—	6.0	7.5
	74	Negative	5.5	Positive	Positive	Unknown	—	—	5.0
	74	Negative	1.0	Unknown	Unknown	Unknown	—	9.0	8.0
	45	Positive	2.0	Negative	Negative	Diploid	9.0	9.0	9.0
	84	Negative	2.0	Unknown	Unknown	Unknown	—	9.0	9.0
	74	Positive	3.0	Unknown	Unknown	Aneuploid	4.5	6.0	5.0
	67	Positive	3.0	Negative	Negative	Aneuploid	—	6.0	6.0
	43	Negative	1.5	Negative	Negative	Aneuploid	—	—	9.0
	78	Negative	1.0	Unknown	Unknown	Unknown	3.0	—	—
	71	Unknown	3.0	Negative	Positive	Unknown	—	9.0	9.0
Lobular carcinoma	40	Negative	4.0	Negative	Positive	Diploid	5.0	—	—

DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; PR, progesterone receptor.

[a] These two slides represent tissue from the same tumor. On one slide the benign glands were poorly preserved and not scored whereas on the other slide, the invasive cancer was poorly preserved and not scored.

TABLE 3. Staining of Slides for Thymosin β15 Grouped by Diagnosis

Primary diagnosis	Number of slides	Number not scored	Average score	Percentage positive
Fibroadenoma	9	0	4.6	33
Fibrocystic disease	1	0	3.0	0
Sclerosing adenosis	5	0	4.2	40
Atypical hyperplasia	6	1	4.4	40
All benign	21	1	4.3	35
DCIS	6	1	6.2	100
Infiltrating ductal carcinoma	15	2	7.1	92
Infiltrating lobular carcinoma	1	1	—	—
All infiltrating cancer	16	3	7.1	92
All malignant	22	4	6.9	94
Total	43	5	5.5	70

DCIS, ductal carcinoma *in situ*.

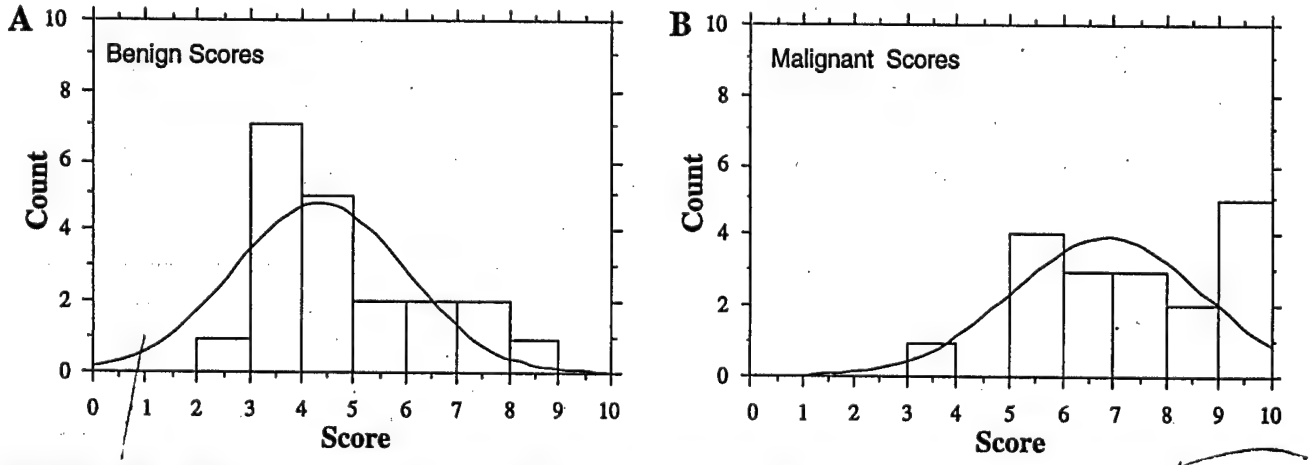

FIGURE 3. Distribution of cases by score of thymosin β15 staining in benign (A) and malignant (B) breast tissue. The slides were then scored by three observers for intensity of staining of the breast epithelium, and the combined scores are shown grouped by benign (n = 20, **histogram A**) or malignant (n = 18, **histogram B**) primary diagnoses. Each ***bar*** on the histograms represents the number of cases with a score ~~in a range~~ that ~~includes~~ the number on the axis at its left margin and the number on the axis at its right margin. Each ***curve*** represents a normal distribution ~~of the included cases~~ with the same mean and standard deviation ~~derived from the scores~~ on the histogram. The histograms show that the score of 5 seems to separate the benign from the malignant cases, with the majority of benign cases scoring less than 5 and all but one of the malignant cases scoring 5 or greater.

TABLE 4. Contingency Table of Benign or Malignant Slides *versus* "Positive" or "Negative" Staining for Thymosin β15

	Negative	Positive	Totals
Benign	13	7	20
Malignant	1	17	18
Totals	14	24	38

χ^2 = 14.387; Fisher's exact *P* value = .0002.

nancy was toward increased thymosin β15 expression. It is also likely that increased levels are associated with physiologic epithelial remodeling. Because the breast is an organ that undergoes continual remodeling (associated with the menstrual cycle), it is not surprising to see this that motility-related protein is expressed in some benign tissues. Expression in that context could obscure the changes associated with malignancy.

The diagnostic utility of the increased thymosin β15 expression seen in malignancy remains to be assessed. This work, though provocative, has insufficient numbers and follow-up to evaluate the independent prognostic value of this marker. Work is underway to produce a monoclonal antibody that will be used in future studies. In summary, this study shows that within breast epithelium, thymosin β15 is localized to the cytoplasmic compartment, as is the case with thymosin β4 and thymosin β10 (8), and that thymosin β15 is upregulated in malignant (compared with benign) breast tissue. Future studies are planned to evaluate the precise role and prognostic value of thymosin β15 in breast disease.

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Expression Of *c-met* Is A Strong Independent Prognostic Factor In Breast Cancer

Running Title: *Met* expression in Breast Cancer

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Precis

Strong expression of *Met*, the receptor for hepatocyte growth factor, in invasive ductal carcinoma of the breast predicts significantly worse 5 year survival, independent of metastases to lymph nodes or steroid hormone receptors.

Key Words: adhesion, Scatter Factor, Hepatocyte Growth Factor (HGF) receptor, survival

Abstract

Background: The *c-met* proto-oncogene encodes the *Met* protein, the receptor for scatter factor/hepatocyte growth factor (SF/HGF), a growth factor that modulates the motility and stable interaction of the epithelial cells. This study assesses the expression of *Met* receptor in breast carcinoma and its prognostic value with respect to survival.

Methods: Immunofluorescence was used to evaluate 91 archival breast cancer specimens using a polyclonal antibody to the cytoplasmic domain of the receptor. Cases were scored by 2 pathologists on a percentage basis then converted to binary scores (positive or negative) on the basis of a bimodal distribution.

Results: Strong expression of *Met* was found in 20 cases (22%) of invasive ductal breast tumors. The five year survival in the in patients whose cancers showed decreased *Met* expression was 89%, in contrast to a 52% five-year survival rate in patients whose cancers expressed *c-met* (p=0.008). This trend is also seen in patients without lymph node metastases at presentation where *Met* negative cases showed 95% five year survival compared to only 62% for *Met* positive cases (p=0.006) Multivariate analysis using the Cox Proportional Hazards Model showed *Met* expression is an independent predictor of survival with predictive value nearly equivalent to that associated with lymph node status.

Conclusions: We conclude that expression of *Met* in invasive ductal carcinoma of the breast is a strong, independent predictor of decreased survival and may be a useful prognostic marker to identify a subset of patients with more aggressive disease.

Introduction:

The process of tumor progression, from its inception to advanced terminal stages, results from a multi-step process including activation of oncogenes and inactivation of tumor suppressor genes. Detection of mutation or amplification of tumor related genes was thought to be a potentially valuable prognostic indicator to augment the more traditional prognosticators, tumor size, node status and histologic type or grade[1, 2]. Unfortunately, the predictive value of most of these new markers as been less than that achievable with the conventional standards. As a result, there is no useful standard for prediction of recurrence in patients with no nodal metastasis at presentation.

To have prognostic value, a new marker must have predictive value as strong as, and independent of, the current standards. The receptor for Scatter factor/Hepatocyte Growth factor (SF/HGF), *Met* may be an example of such a marker. SF/HGF is a mesenchymal cell-derived protein that dissociates epithelial cell colonies by causing a breakdown of intercellular junctions, scattering contiguous sheets of cells , and appears to modulate cell motility and invasion[3-8]. The *c-met* proto-oncogene product has been identified as SF/HGF receptor [9]. The *Met* receptor is selectively expressed in several normal human epithelial tissues as well as in carcinoma [10-12]. The c-*met* encoded receptor is a 190 kD glycoprotein consisting of a transmembrane 145 kD β subunit and an extracellular 50 kD α subunit [13] and is in the tyrosine kinase family of receptors[8]. The ligand-activated cytoplasmic domain of the *Met* receptor is responsible for transducing the signal for cell dissociation, motility and mitogenesis [3] and has recently been specifically associated with tubulogenesis [14].

Consideration of SF/HGF as a prognostic marker was initially stimulated by the finding of immunoreactive HGF in tissue extracts from primary human breast cancer but not in human

breast cancer cell lines [15]. Investigation of the level of expression of HGF message [40] and protein in breast carcinoma has been done [41] and it has shown to be a significant independent factor in predicting survival [16]. Since breast cancer cells respond to SF/HGF as a mitogen, they must express the *Met* receptor. The expression of *Met* in breast tissue [17] and breast carcinoma has been demonstrated[18] and correlated with relapse of the disease [19]. Two groups have examined both HGF and *Met* in benign and malignant breast tissue and shown that there is frequently co-expression in carcinoma [20,42]. Furthermore, they found that both are overexpressed in breast tumors, as compared to benign lesions. Other sites have also been examined and it appears that *Met* expression may have prognostic value in gastric cancer where expression correlates with higher stage and grade tumors [21] and in prostate cancer where it is more frequently expressed in metastatic tumors than primary lesions [22].

To evaluate the independent prognostic significance of expression of the *Met* protein in breast carcinoma, we studied *Met* expression in 91 breast cancer patients. In this study, we show the relationship of *Met* expression with age, size of tumor, nodal involvement, estrogen receptor (ER) status, and survival.

Materials and Methods

Polyclonal antibodies. The "*c-met*" polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz , California) is affinity purified rabbit IgG antibody against the synthetic peptide corresponding to the last 12 amino acids at the carboxyl terminal of human *Met* protein.

Patient population. The cohort included 91 patients who have had breast resections at Yale-New Haven Hospital for invasive ductal breast cancer. Approximately half of them presented with metastases to regional lymph nodes. The age at diagnosis ranged between

26 and 88 years (average 58.1) and survival time ranged from 39 days to 14.1 years (average 5.4 years, median 5.1 years). The cohort showed an 88% 10-year survival of node negative cases and 45% 10-year survival of node positive cases, which compares well with the literature[23] and suggests that this is a representative population. Clinicopathologic parameters collected on each case included age, tumor size, tumor histologic grade, estrogen receptor status, lymph node involvement, and survival . Other parameters including progesterone receptor, ploidy, histologic and nuclear grade were collected, but these parameters were available on less than 40 cases and thus are not included in the analysis. All material was collected under the auspices of the Critical Technologies Program at Yale and in accordance with human investigation committee protocol #8219 to the principal investigator (D.L.R.).

Immunostaining. Sections were obtained from 91 cases of invasive ductal breast carcinoma. Additional sections were derived from 10 cases of benign breast tissue specimens for control. Standard histologic sections were cut from formalin-fixed paraffin-embedded blocks and baked at 60° C overnight. Sections were deparaffinized with xylene, rehydrated and then blocked with a mixture of methanol and 30% H_2O_2 and prepared for immunostaining using a pressure cooker antigen retrieval method [24]. Overnight incubation was performed at 4 C in a humidity tray with a 1:250 dilution of the polyclonal *c-met* primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 0.3% bovine serum albumin (BSA) in Tris-buffered saline (TBS). Following incubation, slides were washed 7 times with TBS including 0.01% triton X-100 in the 6th wash. For increased sensitivity and better subcellular localization, Cy3-conjugated second antibodies were used instead of the conventional enzymatic reaction-based chromogens. Cy3-Goat anti-rabbit antibody (Jackson ImmunoResearch Labs, West Grove, PA) was diluted 1:500 in TBS with 0.3% BSA and incubated for one hour before washing, as above, and coverslipping. Slides are

stored at -20°C to maintain the fluorescent signal, which appears to be stable for over one year.

Histologic scoring and analysis. Cases were examined on an Olympus AX-70 epifluorescence photomicroscope by two pathologists and scored 3 times (twice by R. A. D. G. and once by D.L.R.) as described below. In each case, a serial hematoxylin and eosin section from the same block was examined for orientation and confirmation of the histologic diagnosis. Each case was scored blindly with respect to patient history, presentation and previous scoring. Staining for *Met* protein was evaluated in the tumor as well as in the normal breast, when present in the section (about 80% of the cases). A tumor sample was scored by the percentage of epithelial cells staining for *Met* protein only if intense cytoplasmic and/or membranous reactivity was seen. No attempt was made to quantify intensity of staining. There was a clear, reproducible and easily definable bimodal separation. Cases were either less than 20-30% positive or greater than 70-80% positive. The 10 cases from the benign breast tissue and the lymph nodes were scored as controls and all were positive.

For analysis, we broke this study into 2 groups; *i.* Positive for expression and *ii.* Negative for expression (as discussed above). Using this scoring system, there were no discrepancies in scoring between the 2 pathologists. Splitting of the negatives into smaller groups was attempted, but no statistically significant differences were found and these smaller splits were less reproducible. In six lymph node positive cases, tumor was also examined in the lymph nodes. In each case the *Met* expression score in the tumor in the node was identical to that of the primary breast lesion.

Statistical analysis: Patient follow-up information was obtained from Dr. Diane Fisher in conjunction with the Yale Comprehensive Cancer Center and the Connecticut Tumor

Registry. The association of various disease parameters was analyzed by chi-square test. The relapse-free survival and overall survival curves with or without node involvement were generated by Kaplan-Meier method. The Cox proportional hazards model was used to assess independent prognostic value of each variable. All calculations were made using the StatView 4.5 Software on a PowerPC Mac 7100.

Results and Discussion

Previous studies have used this *c-met* primary antibody in immunostaining experiments, and it has demonstrated specificity for *Met* protein [8, 25]. In our own studies (figure 1), the antibody recognizes a 145 kD band in lysates from breast cancer cell lines, with light bands at 190 and 170 kD (precursors). This is consistent with other description of the mobility pattern by other investigators [10].

In benign breast tissue cases studied (fibrocystic change, fibroadenoma, and normal ductal and lobular tissue) moderate to intense *Met* staining in ductal and lobular epithelium was seen in all cases (figure 2a,b) with staining occurring in essentially all cells within a duct or lobular unit. Expression was present in the membrane and cytoplasm of the breast epithelium and absent in the stroma. Most cases of infiltrating ductal carcinoma showed marked loss of expression of *Met* with <5% of cells showing any immunoreactivity. Some cases showed expression in 5-15% of the cells, distributed randomly throughout the tumor. Nearly all cases, including many of those that were completely negative, showed expression in adjacent normal ducts (figure 2c,d), serving as an internal positive control. Subdivision of cases by percentage of cells stained was examined and the distribution was clearly bimodal, so for purposes of scoring cases were divided into two groups, positive or negative. Example of each illustrate typical negatives (figure 3a) with less than 5% of cells actually staining and typical positives (figure 3b) with nearly all (>90%) cells staining. No attempts were made to quantitate or analyze intensity of staining or subcellular localization in this study.

Overall, 20 of the 91 (22%) invasive ductal breast cancers showed strong (positive) expression of the *Met* protein. Distribution of the pattern of *Met* expression in node negative and node positive patients is shown in figure 4. Analysis was performed by the chi-square method to assess association between *Met* positivity and node status, age at

presentation, hormone receptor status and tumor size. No significant correlations were found except between the expression of *Met* and tumor size (p=0.0348) when a tumor size of 3 cm or greater was chosen to separate the two groups.

The relationship between *Met* expression in tumors and survival was assessed using the Kaplan-Meier method. Significantly decreased survival was seen in patients expressing c-met (Figure 5). In the *Met* negative group, survival at 5 years was 89.6% and 72.7% at 10 years, compared to 52% survival at both 5 and 10 years for the *Met* positive group (p=0.008 by Mantel-Cox logrank test, Figure 5A). When these cases were stratified by node status at presentation, both node-positive and node-negative groups showed survival curves suggesting worse prognosis in *Met* positive tumors (Figure 5B and 5C).

To determine the independent predictive value of *Met* positivity and compare it to some established prognostic factors, the Cox (Proportional Hazards) Model was used in both univariate and multivariate tests. The data, summarized in Tables 1 and 2, shows that *Met* has independent predictive value that is equal to or better than that of size or node status. Note that for the multivariate analysis the estrogen receptor variable was excluded as there were only 40 cases on which this value was determined. In a separate chi square analysis, estrogen receptor was not associated with *Met* expression (p=0.99). Since progesterone receptor data was available on less than 40 cases, no analysis was attempted.

Finally, we question whether the poor prognosis associated with *Met* expression in tumors was a result of reactivation of *Met* in some tumors or if expression was never lost. Data from cell lines suggests that the *c-met* gene might be expressed in normals, lost in tumors, then regained as tumors become more aggressive[43]. To assess this, we were limited to only 6 cases where a subsequent resection was done at least 2-3 years after the primary resection scored in this study. We found 2 of 6 negative cases that converted to a positive

phenotype in latter specimens. This data is insufficient to draw any conslusions, but at least it is consistent with cell line data that suggests that *Met* expression may initially be lost, and then reactivated as tumors progress. If this proves to be true, it is possible that the association between *Met* expression and poor survival may be even stronger than that estimated by this study since malignant cells were assessed at only one time point.

The possible re-activation of expression of *Met* makes it an unusual marker. Other breast cancer markers have been seen to be retained, as is the case for steroid receptors[26, 27], or amplified as is the case for HER2/neu [28, 29]. Other phenotypes include increased protein expression seen for epidermal growth factor receptor [30] and P53 [31] or decreased protein expression, as the case for adhesion proteins[32-34]. Although further study is required to quantitate expression levels, *Met* expression may be unique in that it appears to be associated with tumor progression, being present in benign lesions, lost in invasive cancer, and then reactivated or even overexpressed[20] in a set of tumors with a poor prognosis, inferring a more progressive or advanced phenotype.

Since *Met* is expressed in normal tissue[5], it is somewhat unexpected that its expression in tumors is strongly associated with poor prognosis. On the other hand, activation of the *Met* receptor in tissue culture cells by interaction with scatter factor results in dissociation of monolayers and a "scattering" of the cells, giving the epithelial cells a mesenchymal phenotype[3, 35]. Activation of *Met* also activates expression of urokinase and its receptor, enabling cells to degrade extracellular matrix [36]. These properties are consistent with expression in high grade malignancy are less congruent with expression in normal cell. Multiple functions have been attributed to *Met*, depending on its ligand, context of expression, presence of extra-cellular matrix and possibly other factors as well[4]. It is most likely that the *Met* expressed in normal cells transmits different signals, and may interact with different ligands, than the *Met* expressed in high grade tumors. Furthermore, recent

studies suggest that the level of expression may be important. Jin and colleagues in the Rosen lab show that overexpression of both *Met* and scatter factor occurs in breast tumors when compared to normal tissue [20]. Although we did not attempt quantitation in this study, we also observed that when *Met* was expressed in tumor cells, they were generally brighter than adjacent benign ducts.

A large number of potential prognostic markers for breast cancer have appeared in the literature[37, 38], but very few are proven. Typically markers may appear promising in early studies, but when rigorous statistical methods are applied, markers prove either ineffective or dependent on other known prognostic markers. Thus, relatively few standard markers including size, histologic type, nuclear and histologic grade, lymph node status, and ER/PR status have evolved as the recommended markers[39,23]. In that light, we present this work as a promising first study that can claim independent prognostic value in testing by the Cox Proportional Hazards Model and has predictive value with respect to survival, even in node negative cases. This work is supported by two previous studies addressing *c-met* and survival, one showing independent predictive value related to HGF/SF levels [16] and another showing LOH at the *c-met* locus is not associated with traditional markers but is predictive of decreased survival [44]. Furthermore, the simplicity of the technique and the general availability of the required reagents makes this any easy assay for any pathology lab. Further studies are underway, with emphasis on node negative patients, to evaluate the prognostic significance of *c-met* expression.

Dedication:

This work is dedicated to patients with breast cancer and their families, including relatives of the authors, Ruthy and Robin Brown, Barbara Rimm and Mary Ruth Reitz.

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Figure Legends:

Figure 1. A western blot of 4 cell lines for *Met* and β-catenin shows that the antibody used recognizes a 145kD band, as expected for the β subunit of *Met*. The cell lines used were (1)A431, (2)MCF-7, (3) MB-468, and (4) HBL100. β-catenin, which migrates at 92kD is shown as a control for loading since the levels of expression of *Met* vary in the cell lines chosen.

Figure 2. Sections with normal ducts and lobules show expression of *Met* both at the membrane and diffusely, in the cytoplasm, but not in the nucleus. Figs A and C show hematoxylin and eosin stained images while B and D show serial sections visualizing *Met* reactivity with Cy3 fluorescent staining. The lobular units shown at 400X magnification in A and B show a diffuse cytoplasmic pattern. The larger duct shown at 200X magnification in C and D shows strong cytoplasmic staining and provides a good contrast to the near complete absence of staining of the tumor cells, visualized in the H&E image, surrounding the normal duct.

Figure 3. Illustration of cases of invasive ductal carcinoma with different *Met* expression patterns. H&E (A and C) and immunofluorescent images (B and D) at 200X original magnification are shown as representative cases showing examples of a negative case(A and B) and a positive case(C and D) staining in regions of high cellularity in cases of infiltrating ductal carcinoma. Note this negative case shows some (20%) strongly staining cells and is an example of the highest staining levels still considered "negative". Figure 2D is a more typical negative case with about 5% of cells staining for *Met*.

Figure 4. A frequency distribution of *Met* expression in 91 cases of infiltrating ductal carcinoma of the breast split by *Met* scoring and stratified by lymph node status at presentation.

Figure 5. Kaplan-Meier survival analysis of cases by *Met* expression, overall (A) and split by lymph node status at presentation where (B) is node negative patients and (C) is node positive patients. Inset in the lower left corner of each plot is the p value testing the significance of the difference in the survival curves by the Mantel Cox Logrank test.

Table 1

Univariate Proportional Hazards

Variable	Relative Risk	95% Confidence Interval	p value	n
Met positive	3.34	1.38 - 8.06	0.007	91
Lymph Node positive	4.25	1.42 - 12.6	0.010	91
Age less than 50	1.68	0.69 - 4.00	0.248	91
Estrogen receptor negative	1.84	0.41-8.28	0.427	40
Tumor greater than 3 cm	3.46	1.25 - 9.61	0.017	90

Table 2

Multivariate Proportional Hazards*

Variable	Relative Risk	95% Confidence Interval	p value
Met positive	2.87	1.15 - 7.14	0.024
Lymph Node positive	3.14	1.04 - 9.52	0.043
Age less than 50	1.91	0.79 - 4.65	0.151
Tumor greater than 3 cm	2.33	0.81 - 6.71	0.118

* Estrogen receptor is omitted since only data is available for only 40 cases. For this analysis n=88 since size data was unavailable on 3 of the 91 *c-met* scored cases.

Figure 1

Figure 2

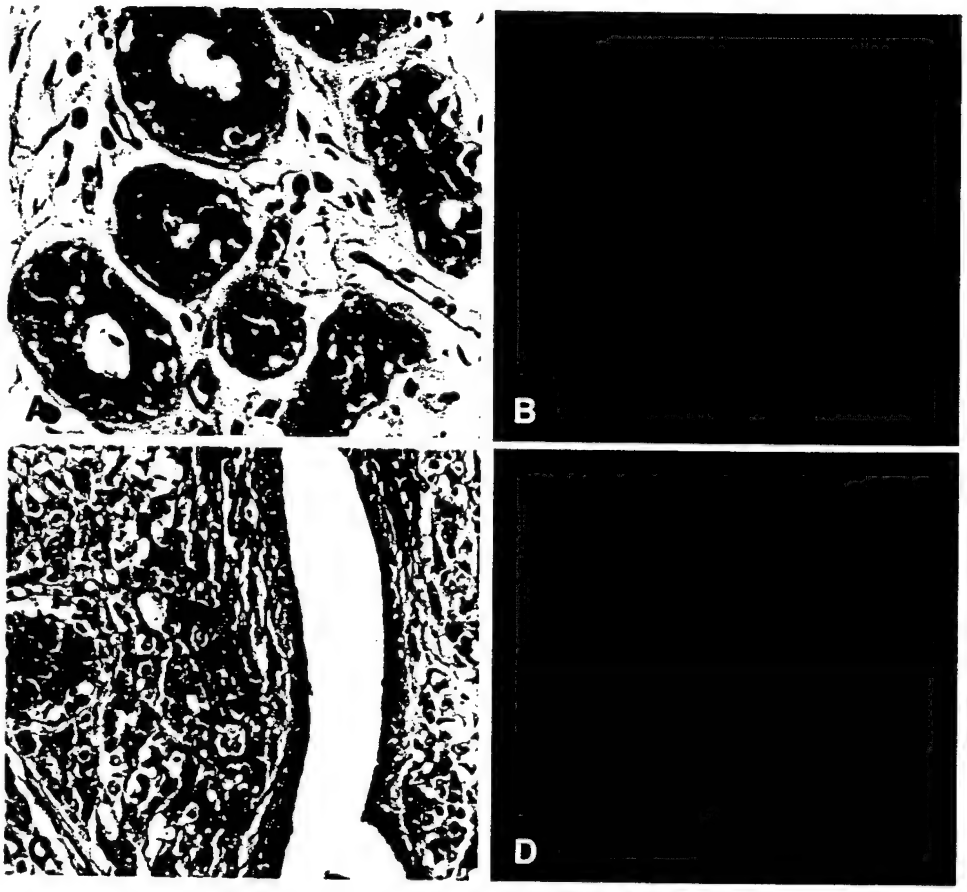
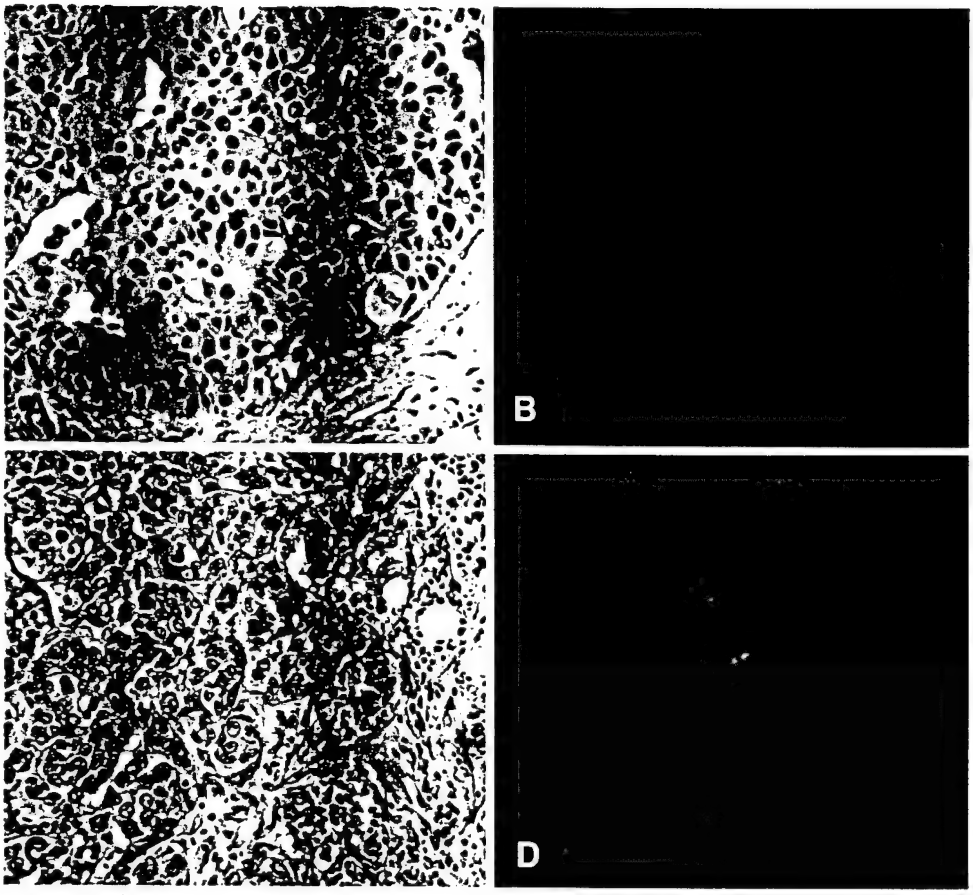


Figure 3



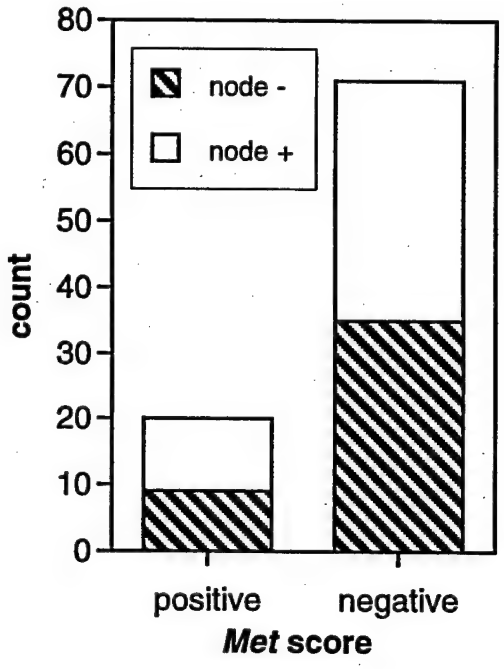


Figure 4
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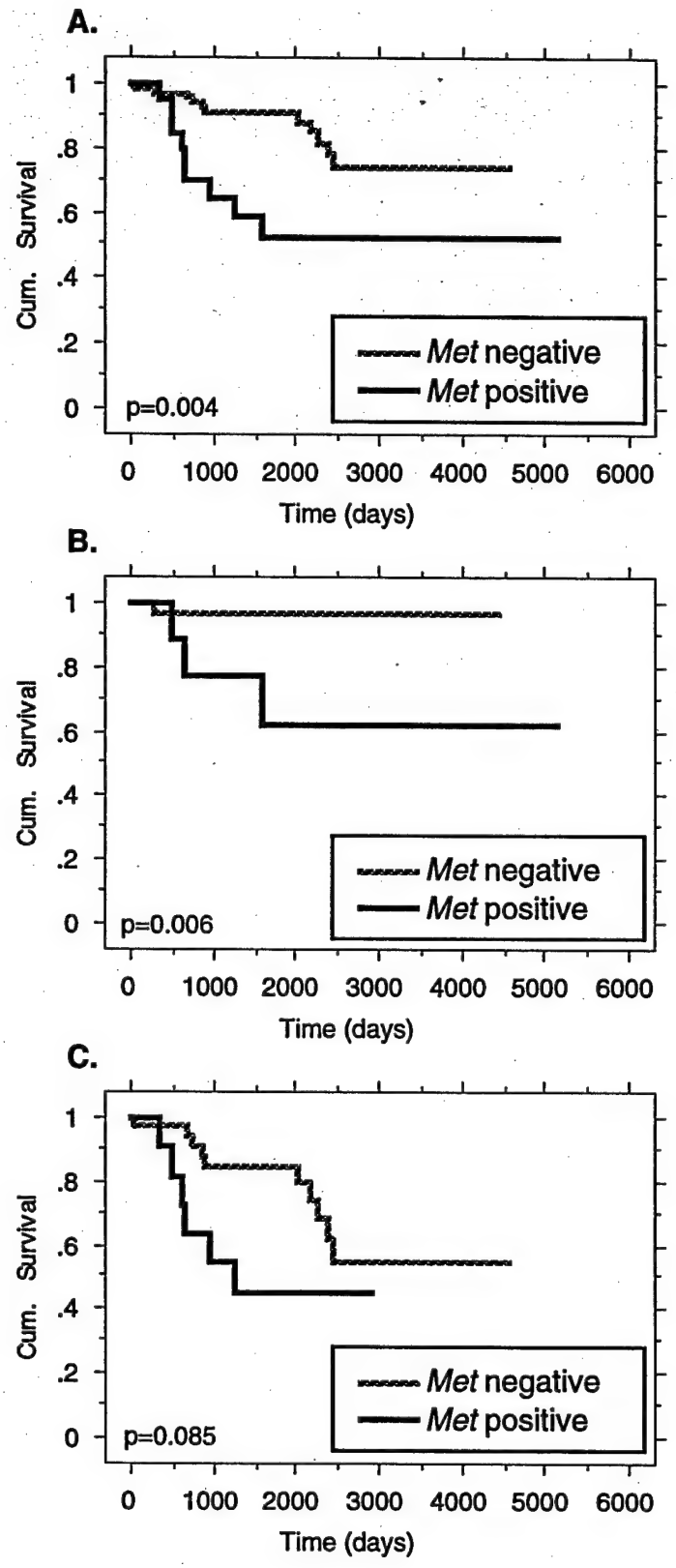

Figure 5
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The expression of p120ctn protein in breast cancer is independent of α- and β-catenin and E-cadherin

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Abstract

Several studies have reported loss or alteration of expression of E-cadherin in breast cancer and more recently changes in levels of expression of the catenins. We use the sensitive method of immunofluorescence to examine E-cadherin, α-catenin, β-catenin, and p120ctn (formerly p120CAS) expression in 91 cases of invasive ductal carcinoma. As expected all 4 proteins co-localize to the junctional regions of the cells. Although nuclear localization has been described for β-catenin in colonic polyps, no examples where found in these breast cancer cases. We find that while alteration is common in the catenins and E-cadherin, complete loss, as exemplified by E-cadherin in lobular carcinoma (where E-cadherin is frequently mutated), is rarely seen. In contrast, the catenin related protein, p120ctn shows an expression pattern that is significantly unrelated to the other catenins (or E-cadherin) including complete loss of expression in about 10% of the cases. No statistically significant correlations with traditional prognostic indicators were observed with any of these proteins. We conclude 1) that expression of E-cadherin, α- and β-catenin are generally retained at the membrane, although frequently reduced or altered; 2) that complete loss of p120ctn expression is seen in about 10% of the cases; and 3) that there is a significant correlation in the expression of E-cadherin and the catenins, but no correlation between these molecules and p120ctn, suggesting an absence of coordinate regulation.

Introduction

Many studies have been done examining levels of expression of adhesion associated markers with the goal of identifying an association with metastasis. Epithelial cell-cell adhesion is primarily mediated by E-cadherin (see [1] for review) and its associated cytoplasmic proteins, the catenins (see [2] for review). E-cadherin expression is frequently altered in both ductal and lobular carcinomas [3; 4; 5; 6; 7] and E-cadherin gene mutations have been detected in lobular carcinoma [8; 9; 10]. Alterations in expression of α-catenin [11; 12] and also β-catenin and plakoglobin [7; 13] have been reported in breast cancer and breast cancer cell lines [14]. Although no correlation with survival has yet been shown for the catenins, one study has recently suggested that E-cadherin alterations may be correlated with decreased disease free survival [6]. Other studies have shown no correlation [15; 16; 17].

Expression of the catenin-related protein p120ctn (formerly p120CAS) has not yet been examined in human breast tissue. This protein was first discovered as a major substrate of the src tyrosine kinase [18], and several receptor tyrosine kinases, including the EGF, CSF-1 and PDGF receptors[19][20]. More recently, p120ctn has been defined as a member of the cadherin-based cell-cell adhesion complex[21; 22; 23]. P120ctn contains a series of 42 amino acid *armadillo* repeats placing it in the *arm* family with other cadherin associated proteins β-catenin and plakoglobin [24]. Further studies showed p120ctn binds directly to E-cadherin, but unlike plakoglobin and β-catenin, it does not bind to either α-catenin or the APC protein [25]. Biochemical studies suggest that p120ctn interacts with E-cadherin at a different site from the β-catenin/plakoglobin binding site [21]. In addition, p120ctn coprecipitates with other members of the classical cadherin family (like N- and P-cadherin) suggesting that the interaction is broadly applicable to cadherin biology. Although its exact function is still unknown, it may play a role in modulation of adhesion since the

association of the tyrosine phosphorylated p120ctn with E-cadherin is elevated in *ras* transformed breast epithelial cell lines [26].

Since p120ctn plays a role in modulation of adhesion, it may be another candidate for assessment of the metastatic potential of tumors, as down regulation of adhesion is a primary event in metastasis[27; 28]. Unlike other adhesion proteins where there is loss of expression in tumor cell lines[14; 29], p120ctn shows heterogeneous expression patterns, with isoform variability but, as yet, no evidence of complete loss of expression [30]. Several murine isoforms of p120ctn have been defined [30], and at least 6 human isoforms are currently in the process of characterization (Frans Van Roy, personal communication). This isoform diversity is more complex than other cadherin-associated proteins of the adhesion complex but alternative splice forms have been described for both β-catenin [31] and α-catenin [32].

In this study we compare expression of p120ctn to E-cadherin and the conventional catenins. Tissue sections were labeled with Cy3-conjugated fluorescent antibodies, rather than conventional enzyme conjugated techniques to increase sensitivity to the point at which loss can be reliably distinguished from alteration, or decreased intensity, as is frequently seen for these adhesion related proteins. To calibrate our assay, we use 10 cases of lobular carcinoma as a reference for true loss of E-cadherin expression. Confirming the more recent literature, we find alteration of E-cadherin and α- and β-catenin common, but true loss extremely rare. This pattern is different from that seen for p120ctn where there is true loss in about 10% of the cases (as defined by loss of reactivity of multiple monoclonal antibodies). Furthermore, we find colocalization, but no correlation in level of expression of p120ctn and the other adhesion proteins examined, suggesting independent regulation.

Materials and methods:

Tissue Acquisition and Study Population: The cohort included 91 patients who have had breast resections at Yale-New Haven Hospital for invasive ductal breast cancer. Approximately half of them presented with metastases to regional lymph nodes. The age at diagnosis ranged between 26 and 88 years (average 58.1) and survival time ranged from 39 days to 14.1 years (average 5.4 years, median 5.1 years). The cohort showed an 88% 10-year survival of node negative cases and 45% 10-year survival of node positive cases, which compares well with the literature[33] and suggests that this is a representative population. Clinico-pathologic parameters collected on each case included age, tumor size, tumor histologic grade, estrogen and progesterone receptor status, lymph node involvement, and survival . This population is ethnically and racially diverse although, due to the nature of the disease, includes only females. All material was collected under the auspices of the Critical Technologies Program at Yale and in accordance with human investigation committee protocol #8219 to the principal investigator (D.L.R.). Ten cases of lobular carcinoma were also selected at random to use as controls for loss of cadherin staining. No clinical information was obtained on this group.

Antibody Preparation: Recombinant fusion proteins were prepared from full-length human cDNA clones for both α-catenin and β-catenin by expression in glutathione-S-transferase (GST)-based expression vectors (Pharmacia, Piscataway, NJ). Each was purified on a glutathione affinity matrix and antisera were raised in rabbits by injection in complete Freund's adjuvant. Antibodies were affinity purified in two steps. Anti-GST activity was depleted by passage over a column of Affi-gel linked to GST. The eluent was subsequently passed over an Affi-gel column with bound α-catenin or β-catenin. After washing, antibodies were eluted with 100 mM glycine-HCl, pH 2.5. Fractions containing active antibodies as detected by enzyme-linked immunosorbent assay (ELISA) were pooled, dialyzed into phosphate-buffered saline containing 1mM sodium azide and stored at

-20°C. A commercial monoclonal antibody to E-cadherin was used (Transduction Laboratories, Lexington, KY).

The p120ctn-specific monoclonal antibodies (Mabs) 12F4 and 15D2 were chosen for their excellent reactivity with formaldehyde fixed tissue sections from a panel of p120ctn-specific Mabs prepared previously (Wu and Reynolds, manuscript in preparation). These antibodies bind to different epitopes in the carboxy-terminal 121 amino acids of p120ctn and recognize all known isoforms of p120ctn that can be distinguished by immunoprecipitation and western blotting analysis. Both Mabs were affinity purified on protein-A sepharose columns. For immunostaining experiments, Mabs 12F4 and 15D2 were used at 7 μg/ml and 2 μg/ml respectively. Other p120ctn Mabs, including 9D5, 5A7, 6H11, 8D11, 9B8 (Wu and Reynolds, manuscript in preparation) and pp120 (Transduction Labs), were used on some cases for confirmation of the staining pattern.

Immunostaining. Standard histologic sections were cut from paraffin blocks and prepared for immunostaining using a pressure cooker antigen retrieval method [34]. Each section was baked at 60° C overnight, then deparaffinized and treated for antigen retrieval by immersion in 6.5 mM sodium citrate (pH=6.0) for 5 minutes in a conventional pressure cooker (KMart). Sections were then blocked with 3% BSA in Tris buffered saline (TBS) (150mM NaCl, 20mM Tris pH=8). Monoclonal antibodies were diluted to 2-7 μg/ml and incubated in a humidity chamber overnight before washing 7 times with TBS including 0.01% triton X-100 in the 6th wash. For increased sensitivity and better subcellular localization, Cy3 conjugated second antibodies were used instead of the conventional enzymatic reaction-based chromogens. Cy3-Goat anti-mouse antibody (Jackson ImmunoResearch Labs, West Grove, PA) was diluted 1:500 in TBS with 3% BSA and placed in the sections for one hour before washing as above and coverslipping. Slides

were stored at -20°C to maintain the fluorescent signal, which appears to be stable for over one year using these conditions.

Histologic scoring and analysis: Each slide was examined on at least two separate occasions by at least 2 individuals including two pathologists and a technologist using either a Zeiss Epifluorescence Microscope or an Olympus AX-70 epifluorescence photomicroscope. The expression of each antigen was scored as one of three final groupings. The final grouping used were: *i.* Normal, cases not reproducibly distinguishable from normal, in either pattern or intensity; *ii.* Altered, a broken or discontinuous staining pattern, or a patchy staining pattern, with or without a decrease in intensity; and *iii.* Loss, a complete loss of staining as the predominant pattern in the section examined, comparable to that seen for E-cadherin in negative control cases of lobular carcinoma. In each case, a serial hematoxylin and eosin section was examined for orientation and confirmation of the histologic diagnosis. Each case was scored blindly with respect to patient history, presentation and previous scoring. Patient follow-up information was obtained from Dr. Diane Fisher in conjunction with the Yale Comprehensive Cancer Center and the Connecticut Tumor Registry. Data analysis was done using StatView 4.5 for Macintosh.

Results:

The specificities of p120ctn Mabs 12F4 and 15D2 were compared by immunoprecipitation and western blotting (from MDCK cell lysates) to the previously characterized Mabs 2B12 and pp120 (from Transduction Labs, Lexington KY) (Figure 1). Mab 2B12 specifically recognized the so called CAS1 isoforms due to the location of its epitope in the N-terminal 100 amino acids of p120ctn [21; 30]. This N-terminal sequence is spliced out in many cell types resulting in the faster migrating CAS2 isoforms which are more abundant than CAS1 isoforms in this cell type. Both CAS1 and CAS2 isoforms were recognized by Mabs 12F4 and 15D2, and their staining patterns were similar of those of Mab pp120. In addition to the similar immunoprecipitation and western blotting patterns illustrated here, the properties of Mab 15D2 are nearly identical to that of Mab pp120 in every parameter tested to date including immunofluorescence, species cross-reactivity, and isoform specificity. Mab 12F4 was chosen for the bulk of the experiments as it showed the best signal-to-noise characteristics in formaldehyde fixed tissues. A selection of 30 cases was examined with 15D2 to confirm the staining pattern. Cases showing true loss of expression were re-examined with multiple monoclonal antibodies to confirm the loss of expression.

The adhesion protein p120ctn is associated with E-cadherin and shows some homology to β-catenin, but its role in stabilization or regulation of adhesion has yet to be defined. Like the catenins and cadherins, the protein localizes to lateral membranes. In breast tissue, it co-localizes exactly with E-cadherin and α- and β-catenin (figure 2). At the resolution of immunofluorescence, the distribution of expression is predominantly basolateral and membranous with pale cytoplasmic expression. In some ductal cells there are increased membrane densities near the apex, presumably correlating with the location of the adherens junction. This finding is more prominent in colonic epithelium than in breast tissue.

Alteration of adhesion molecules in breast cancer has been described frequently in the literature [35]. Alteration or discontinuity of staining is grouped with loss in some studies, but that grouping may overlook a biological distinction. We have observed numerous cases where there are discontinuous or altered staining patterns, frequently with reduced intensity of adhesion protein staining, but true loss is rare. In this study we define a category called "altered" for all cases with a staining pattern that is reproducibly and easily distinguished from normal, but does not meet the criteria for true loss of expression. Generally, normal ducts are present in these cases and provide excellent internal calibration by which to make the judgment of alteration or loss. An example of alteration is shown in figure 3, where tumor surrounds a normal duct. The staining pattern, defined as altered, shows broken and discontinuous, but predominantly membranous staining. In this case the discontinuous α-catenin staining pattern is contrasted with the normal staining in the adjacent duct. The intensity may be reduced compared to normal ducts, but is still easily seen. No attempt is made to quantify intensity.

True loss of expression is defined as a complete absence of antibody reactivity, similar to that seen in the absence of the primary antibody. As a reference for this pattern we examined expression of E-cadherin in 10 lobular carcinoma specimens as mutations in E-cadherin occur in greater that 50% of the cases[9]. We found 7 of 10 selected cases showed complete loss of expression of E-cadherin, as illustrated in figure 4. Again, in each case enveloped normal ducts were used as an internal reference for normal antibody reactivity. Complete loss in the lobular carcinoma cells shows a staining pattern and intensity indistinguishable from the negative control.

Using these criteria for expression, over 100 cases of ductal carcinoma of the breast were examined. Each was scored on the basis of the consensus of at least 2 individuals. The distribution of expression pattern is shown in figure 5. It is notable that complete loss of

expression was never seen for α- or β-catenin and only seen in one case for E-cadherin. All cases showed membranous localization. Nuclear staining of β-catenin as reported in colonic polyps[36] was not seen in any case. In contrast, about 10% of the cases showed loss of p120ctn. To examine correlation of level of expression between proteins as a first assessment of coordinate regulation, the cases were analyzed using the Chi Squared Test. We found highly significant correlations between E-cadherin and α- and β-catenin, but no correlation between p120ctn expression level and any of these adhesion proteins (table 1). That is, frequently cases with "normal" E-cadherin and catenins showed "altered" p120ctn or visa-versa.

The lack of correlation between p120ctn expression levels and the other proteins led to re-examination of these cases. True loss, as seen in 10% of the cases, was similar to loss of E-cadherin expression observed in lobular carcinoma. Normal staining benign ducts were seen surrounded by nests of tumor cells with complete absence of p120ctn reactivity (figure 6). These cases were recut for examination with other monoclonal antibodies to confirm loss. Monoclonal antibodies including 5A7, 6H11, 8D11, and 9B8 showed a pattern of loss indistinguishable from 12F4. The 15D2 antibody showed a pattern of high background, with cytoplasmic staining but no true membranous staining as seen in the adjacent non-neoplastic ducts. A similar pattern was seen with 9D5 and in some cases with the pp120 antibody from Transduction Labs.

Finally, attempts were made to correlate alterations in expression with other known prognostic markers in breast cancer including lymph node status, tumor size, steroid receptor expression, and age as well as overall survival. Although there were some general trends suggesting an association between alteration and poor prognostic factors, no statistically significant associations were found. Similarly, alteration of expression of any of these proteins was not associated with decreased survival. Since only p120ctn showed

complete loss of expression, we compared this to a combined group of altered and normal expression. We again found no correlation with conventional prognostic markers. Since only 8 patients fell into this group, if was difficult to meaningfully address survival. Kaplan Meier analysis suggests that there may be an association between loss of p120ctn and decreased 5 year survival, but statistical significance is not achieved (figure 7).

Discussion

Although this is one of the first studies assessing expression of p120ctn in tumors, many studies have examined the other adhesion molecules. Our findings with respect to those studies generally confirm that alteration of expression is a frequent finding. We find 1) alteration of expression is common in invasive carcinoma but membranous localization is retained, even for β-catenin, in all cases; 2) true loss of expression of E-cadherin, α-catenin and β-catenin is very rare in ductal carcinoma of the breast; 3) The pattern of expression of E-cadherin, α-catenin and β-catenin are highly correlated within each case, but do not correlate with expression of p120ctn; 4) there appears to be complete loss of p120ctn in about 10% of the cases, although we do not yet know if this is a function of genetic mutation or down-regulation; and 5) there are general trends correlating alteration of expression of these proteins with traditional poor prognostic markers, but none are statistically significant or independently predictive.

Although use of immunoperoxidase is an accepted standard approach, by using immunofluorescence we attempt to achieve increased sensitivity. This technique also allows more specific subcellular localization. As the role of β-catenin in signal transduction has become better defined[37], localization to the nucleus is suggested, and has been described in colonic polyps[36]. In invasive ductal carcinoma, nuclear localization is not seen, rather each case shows membranous localization, although discontinuity and decreased intensity are seen in many cases. In general, we believe alteration is a function

of loss of normal differentiation. The discontinuous staining pattern is accounted for by loss of well-defined cell-polarity in many tumors. By analogy to observations in the colon, it may be that earlier lesions (ductal carcinoma in situ or atypical ductal hyperplasia) need to be examined for nuclear expression of β-catenin, or it may not occur at all in breast cancers.

Our finding that true loss of expression is rare or absent in ductal carcinoma has been observed by others[4][9]. Although loss of expression is reported in the literature, it is often grouped with decreased expression [5; 16] and generally less sensitive methods (immunoperoxidase) are used which may be unable to distinguish reduction from true loss [6]. Molecular analysis to detect mutations in these genes also suggests loss due to mutation will be rare as mutations in the E-cadherin gene have never been found in ductal carcinoma [9]. The catenins are less well studied, although preliminary evidence suggests that their loss due to mutation will also be rare in human tumors[10], even though loss of expression has been described in a number of cell lines[14].

In a previous study using a panel of breast cancer cell lines there were no examples of loss of expression of p120ctn [30]. In contrast, this study shows some clear examples of complete loss of p120ctn in breast cancer tissue sections. One explanation of this finding could be an isoform switch resulting in loss of many epitopes. This seems unlikely because previous studies indicate that Mabs 15D2 and 12F4 recognize all known isoforms of p120ctn [30]. An alternative explanation is that the biological processes that are occurring in the tumors with loss of expression represent a stage in the multi-step process of carcinogenesis that is not recapitulated by any cell line yet tested; it is possible that cell lines will eventually be found that show complete loss of p120ctn.

The expression pattern and potential loss of p120ctn has not been previously studied in human breast cancer tissues. Decreased levels of p120ctn expression have been reported in colonic carcinoma ([38] and preliminary data in our laboratory), bladder cancer[39] and more recently in adenomatous polyps[36]. Our study of breast tissue shows reduction or alteration, similar to the colonic cases, but also true loss of expression is seen in a small percentage of the cases. It is unknown whether this loss represents mutation or simply transiently extinguished expression. The fact that some of our cases show areas of loss and other areas with reduction or alteration, suggests that the absence of expression may be a result of down-regulation. As the factors that regulate expression of p120ctn are not well understood, we can not test these cases to distinguish these possibilities. However, consistent with the study in bladder cancers[39] and studies on transformed L-cell lines [40], it appears that p120ctn is not coordinately regulated with E-cadherin and α- and β-catenin. Future studies will attempt to address the possibility of down-regulation versus mutation in these cases.

Acknowledgments:

This work is dedicated to patients with breast cancer and their families, including relatives of an author (DLR) Ruthy and Robin Brown, Barbara Rimm and Mary Ruth Reitz.

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Figure Legends

Figure 1. Specificity of p120ctn-specific monoclonal antibodies. MDCK cell lysates were Immunoprecipitated using the control Mab 12CA5 (control) or the p120ctn-specific Mabs listed across the top. Immunoprecipitates were separated on 8% polyacrylamide gels and then Western blotted with either Mab 15D2 (A) or Mab pp120 (Transduction Labs) (B).

Figure 2. Normal staining patterns of all 4 proteins. The adhesion proteins, E-cadherin, α-catenin and β-catenin show classic baso-lateral membrane staining in normal breast ducts and lobules. As described in other tissues, the localization of p120ctn is essentially indistinguishable from E-cadherin and the catenins. Stains are as described in the methods section and shown at low (20X original magnification) (A-E) and high (80X) power (F-J). Stains, shown beneath each column, are hematoxylin and eosin (A and F), E-cadherin (B and G), β-catenin (C and H) α-catenin (D and I) and p120ctn (E and J).

Figure 3. Example of altered staining of α-catenin in the tumor surrounding a normal duct. Hematoxylin and eosin stains (A and C) at 20X and 80X respectively, show a normal duct surrounded by a poorly differentiated ductal carcinoma. The staining pattern showing α-catenin expression (B and D) at similar magnifications, shows normal staining surrounded by a pattern designated "altered" but not "lost".

Figure 4. E-cadherin staining pattern in lobular carcinoma as an example of true loss of expression. Matched serial sections stained with hematoxylin and eosin (A and C) and anti-E-cadherin visualized with Cy3 conjugated secondary antibodies (B and D) show low and high magnification views of normal small ducts surrounded by malignant cells of a lobular carcinoma. The immunofluorescent frames (B and D) are overexposed to show shadows of the malignant cells with complete absence of E-cadherin expression. The scale bar in A is 100μM.

Figure 5. Distribution of expression of each antigen shows different pattern for p120ctn as compared to the other adhesion molecules.

Figure 6. An example of p120ctn staining showing true loss of expression. Hematoxylin and eosin sections (A and B) show low (20X) and high (80X) magnification views of a small duct lined by benign epithelium, surrounded by the cells of an invasive ductal carcinoma. Frame C shows complete absence of expression of p120ctn in a serial section, with good membranous staining of the entrapped normal duct. The scale bar in frame C is 50 μm.

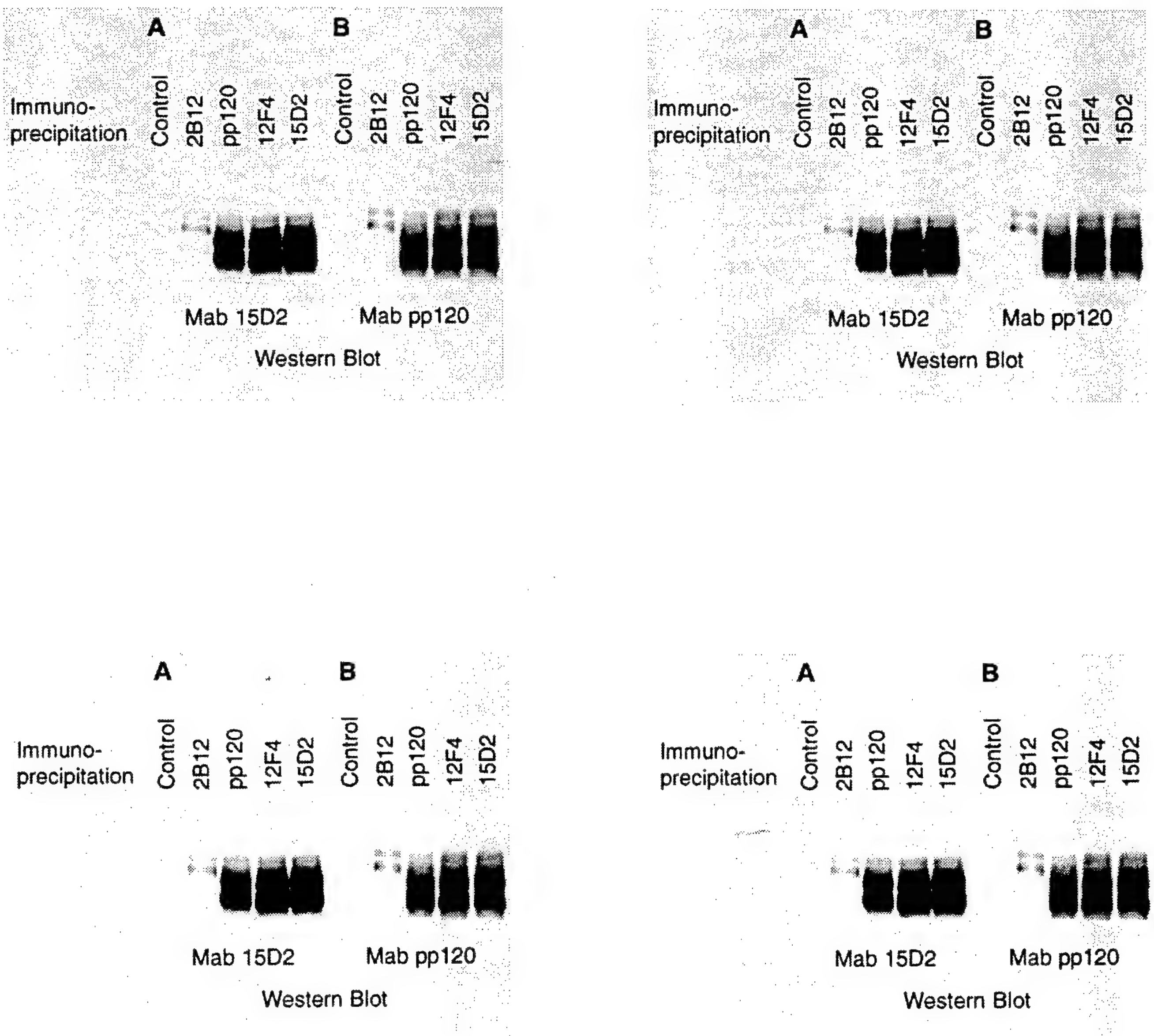
Figure 7. Kaplan Meier analysis suggests loss of p120ctn expression may be associated with poor outcome, but statistical significance is not achieved.

Table 1

High correlation of expression levels is seen between E-cadherin, α-catenin and β-catenin but not p120ctn

	E-cadherin	α-catenin	β-catenin	p120ctn
E-cadherin	xxxxxxxxxxxx	46.3 (p<.0001)	29.2 (p<.0001)	0.36 (p=.547)
α-catenin	46.3 (p<.0001)	xxxxxxxxxxxx	42.2 (p<.0001)	0.68 (p=.408)
β-catenin	29.2 (p<.0001)	42.2 (p<.0001)	xxxxxxxxxxxx	0.04 (p=.835)
p120ctn	0.36 (p=.547)	0.68 (p=.408)	0.04 (p=.835)	xxxxxxxxxxxx

each cell shows the Chi Square value and the corresponding P value in parentheses


A
B
Immuno-
precipitation
Control
2B12
pp120
12F4
15D2
Control
2B12
pp120
12F4
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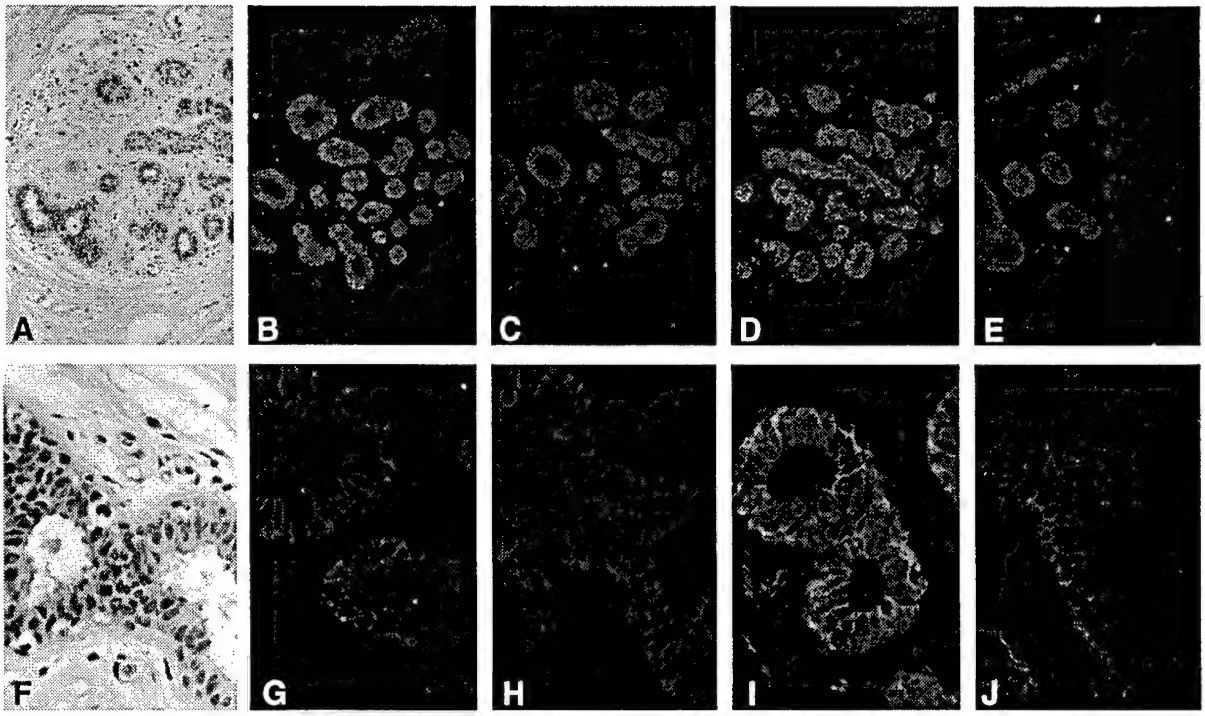
Mab 15D2
Mab pp120
Western Blot

Figure 1



H&E E-cad β-cat α-cat p120CAS

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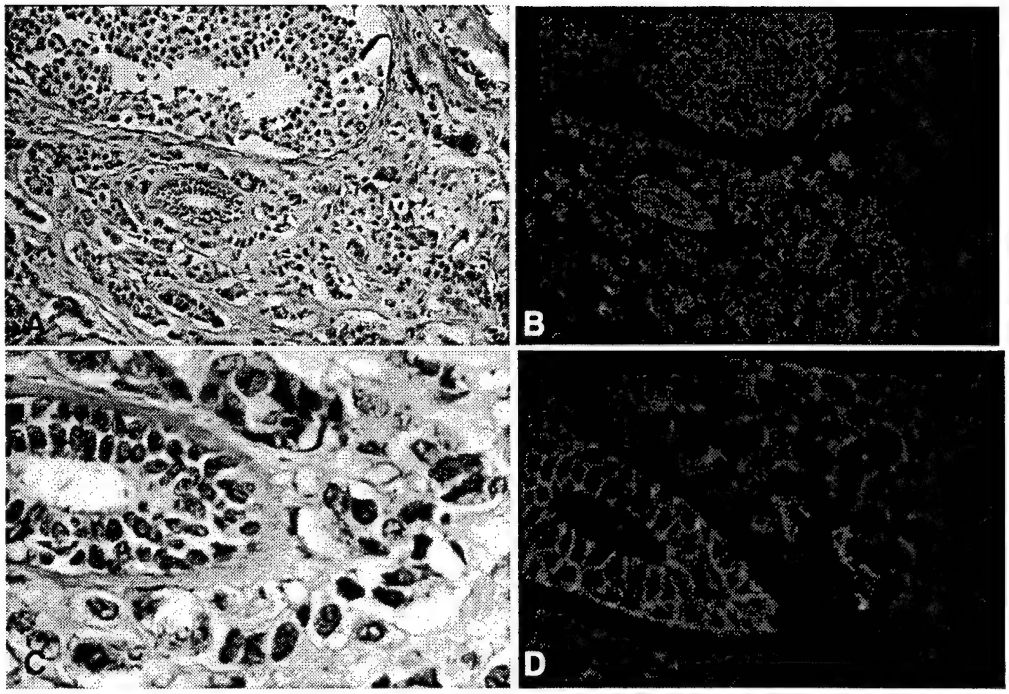
Figure 2



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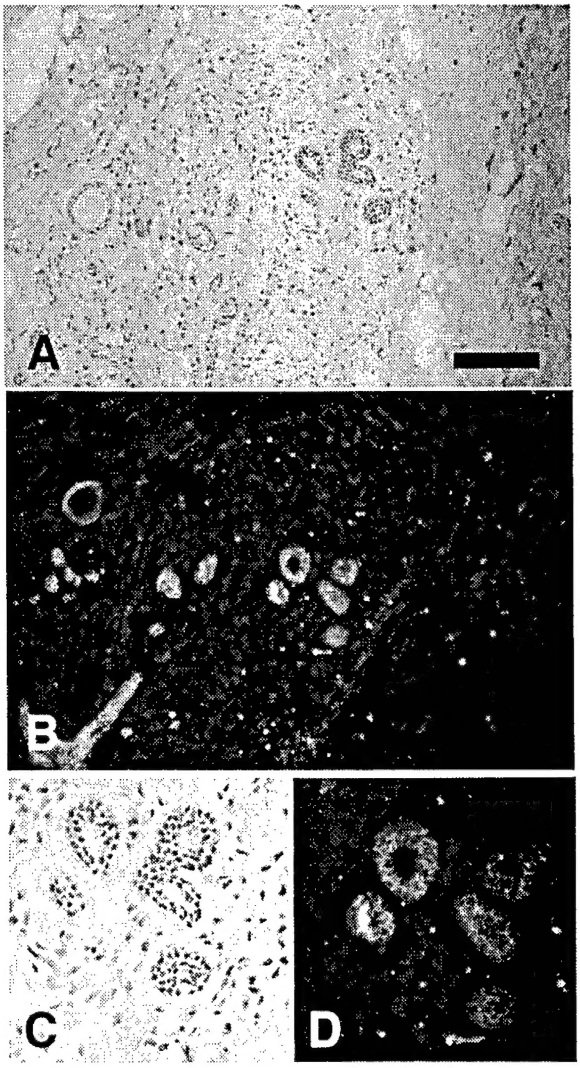

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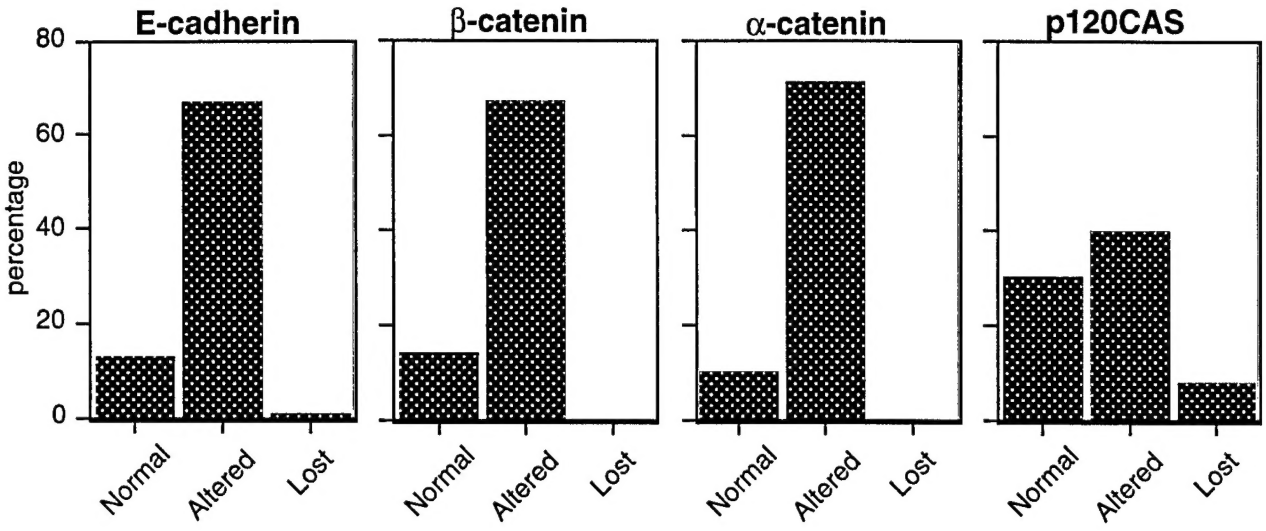

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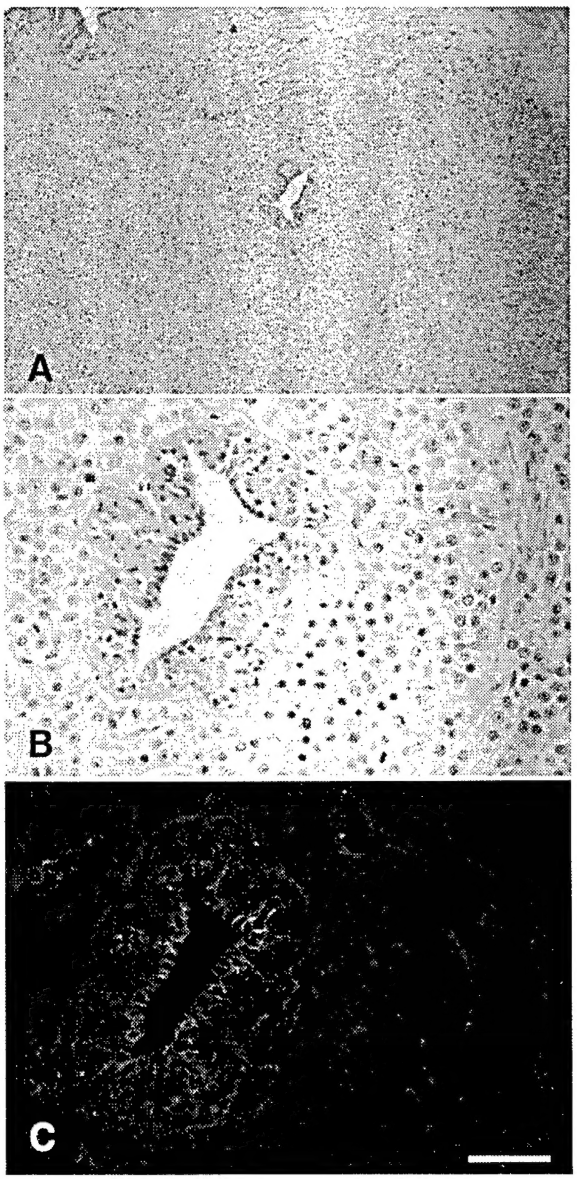

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Figure 6

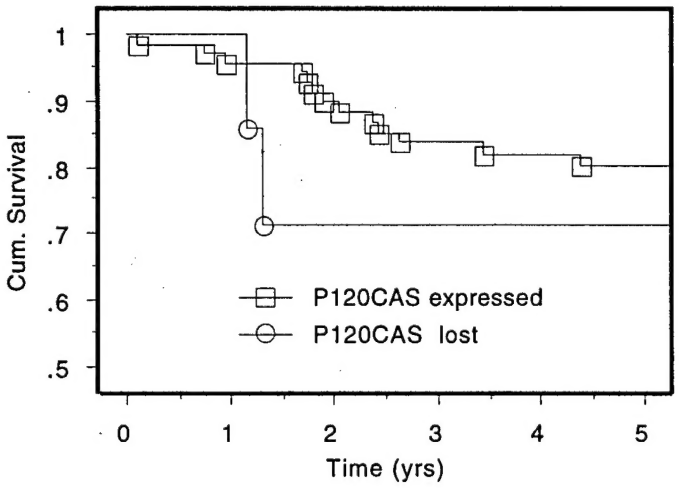

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Figure 7